A THESIS SUBMITTED BY

JOHN BRIAN PRIDHAM

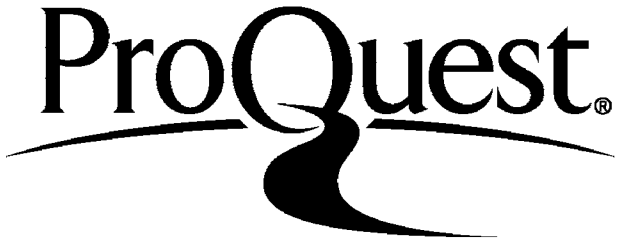
IN CANDIDATURE FOR THE

DEGREE OF DOCTOR OF SCIENCE

OF THE UNIVERSITY OF LONDON

February 1967

Royal Holloway College,
University of London,
Englefield Green,
Surrey.



INTRODUCTION

This collection of 48 publications is divided into three main sections:

A. Original papers and reviews concerning carbohydrates and nucleotides.

B. Original papers and reviews relating to phenolic compounds.

C. Edited books.

The overlap in topics which occurs in Sections A and B is a direct result of the candidate's interest in the borderline field between the three groups of compounds listed.

Publications A1 - A9 are mainly concerned with higher plant polysaccharides and related researches. Prunus gums have been investigated from the points of view of composition, function and biosynthesis. The detailed chemical structures of the polysaccharide moieties have not been emphasized but, instead, certain comparative aspects of the structures of gums arising from different plant tissues. In addition, the minor components of gums have been examined together with the tissues which apparently produce the gums.

The biosynthesis of complex heteropolysaccharides in mature plant tissues was observed for the first time using $^{14}CO_2$ and more recently the biosynthesis of hemicelluloses has been investigated and nucleotide derivatives of L-arabinose and D-xylose shown to be involved.

Methods for the determination of monosaccharides were devised and the dangers of acid catalysed polymerization of these sugars stressed.

Publications A10 (a,b) describe some initial studies with human foetal glycogen. This polysaccharide was shown to be present in foetal livers after only 13½ weeks gestation; structural analysis indicated a close similarity to adult human liver glycogen. Information on the level of some of the enzymes concerned with glycogen metabolism in foetal tissues has been obtained.

Studies with oligosaccharides constitute the main thesis of publications A11 - A19. This work began with the isolation and characterization of the galactose-containing oligosaccharides from broad-bean seeds. The biosynthesis and metabolism of these sugars was later studied: UDP galactose has been implicated in the formation of raffinose in vivo and the enzymes α-galactosidase and UDP glucose: fructose glucosyl transferase (sucrose synthetase) in the breakdown of the trisaccharide during germination. The low molecular weight carbohydrate content and associated enzymes of aspen cambial tissues have also been examined. Reviews on methods in oligosaccharide chemistry are included.

General interest in the stability of glycosidic bonds and also possible methods of making iron-containing organic compounds soluble in water led to the work on α-D-glucosides and acetals of ferrocene derivatives which is described in publications A20 - A23.

Publications B1 - B21 are concerned mainly with the formation and metabolism of phenolic glycosides. In summary, it has been shown that nucleoside diphosphate glucose derivatives are almost certainly involved in phenolic β-D-glucoside formation in plants. The ability to produce glucosides from exogenous phenols has been shown to be a

common characteristic of most higher plant tissues but not of lower plants such as algae and fungi. In higher plants the glucosylating system is rather non-specific with respect to exogenous phenols. This is probably due to the existence of several glucosyl transferases in the tissues. Transfer of D-galactose, D-fucose and D-glucose from 'low energy' glycosyl donors to phenolic hydroxyl groups has been effected using enzymes from microorganisms. This type of transferase utilizing simple D-glucose derivatives apparently does not exist in higher plants. Initial studies suggested that helicin not saligenin was the direct precursor of salicin (further evidence which is unpublished, confirms this).

DOPA metabolism has been investigated. Unexpectedly it was found that only small amounts of this compound are converted to the glucoside in Vicia faba. An enzyme capable of deaminating DOPA with the formation of caffeic acid was detected in several plant species. In DOPA-containing plants which produce melanin the biosynthetic pathway to the pigment cannot be the same as in the animal kingdom as these plant melanins have structures largely based on catechol residues.

Analytical methods have been developed to assist with the studies described above and reviews on phenol chemistry and biochemistry have been prepared.

J. Pridham

2nd Feb '67

CONTENTS

9. A preliminary study on the biosynthesis of hemicelluloses. J.B. Pridham and W.Z. Hassid, Biochem. J., 100, 21P (1966).

10(a) Studies with glycogens from the livers of human foetuses and young children. E.J. Bourne, Anne McLean and J.B. Pridham, Fed. Europ. Biochem. Soc., 1st Meeting Abstracts, A.81, p.65. London, 1964.

(b) The structure and deposition of human liver glycogens. E.J. Bourne, Anne McLean and J.B. Pridham, Biochem J., 98, 678 (1966).

11. Occurrence and metabolism of oligosaccharides in the broad bean (Vicia faba). J.B. Pridham, Nature, 182, 1687 (1958).

12. Oligosaccharides and associated glycosidases in Aspen tissues. J.B. Pridham, Biochem.J., 76, 13 (1960).

13. The separation and identification of oligosaccharides. R.W. Bailey and J.B. Pridham, Chromatographic Rev., 4, 114 (1962).

14. The biosynthesis of galactosylsucrose derivatives. E.J. Bourne, J.B. Pridham and Mary W. Walter, Biochem. J., 82, 44P (1962).

15. Oligosaccharides. R.W. Bailey and J.B. Pridham, Advanc. Carbohydrate Chem. 17, 121 (1962).

16. α-Galactosidase and alkaline β-fructofuranosidase activity in Vicia faba seeds. J.B. Pridham and Mary W. Walter, Biochem. J., 92, 20P (1964).

17. The biosynthesis of raffinose. E.J. Bourne, Mary W. Walter and J.B. Pridham, Biochem. J. 97, 802 (1965).

18. Biosynthesis of raffinose. J.B. Pridham and W.Z. Hassid, Plant Physiol., 40, 984 (1965).

19. Oligosaccharide metabolism in seedlings. J.B. Pridham, Mary W. Walter and H.G.J. Worth, Biochem. J., 100, 22P (1966).

20. The condensation of ferrocenealdehyde with D-glucitol and D-mannitol. A.N. de Belder, E.J. Bourne and J.B. Pridham, J. Chem. Soc., 5486 (1964).

21. β-Glucopyranosides of hydroxymethyl- and hydroxyethyl-ferrocene. A.N. de Belder, E.J. Bourne and J.B. Pridham, J. Chem. Soc., 4464 (1961).

22. Glucosyl derivatives of cyclopentadiene and cyclopentane. A.N. de Belder, E.J. Bourne and J.B. Pridham, Chem. & Ind. 432 (1961).

23. Paper chromatography of ferrocene and its derivatives. A.N. de Belder, E.J. Bourne and J.B. Pridham, Chem. & Ind., 996 (1959).

B. PHENOLICS SECTION

1. Determination of phenolic glycosides and aglycones on paper chromatograms. J.B. Pridham, Anal. Chem., 29, 1167, (1957).

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

3. Paper electrophoresis and paper chromatography of phenolic compounds. J.B. Pridham, J. Chromatog. 2, 605 (1959).

4. The formation of phenolic glycosides by germinating broad-bean (Vicia faba) seeds. J.B. Pridham and M.J. Saltmarsh, Biochem. J., 74 42P (1960).

5. The reaction of carbohydrases with phenolic glucosides. J.D. Anderson, L. Hough and J.B. Pridham, Biochem. J., 77, 564 (1960).

6. Biosynthesis of phenolic glucosides in the absence of UDPG. J.B. Pridham, Chem. & Ind., 1172 (1961).

7. The formation and possible function of phenolic glycosides. J.B. Pridham In "Phenolics in Plants in Health and Disease", (J.B. Pridham ed.) p.9 Pergamon Press, London, 1962.

8. The formation of acidic derivatives of phenolic glucosides by Vicia faba seeds. J.B. Pridham and Margaret J. Saltmarsh, Biochem. J., 82, 44P (1962).

9. The identification of naturally occurring cinnamic acid derivatives. E.J. Bourne, Norma J. Macleod and J.B. Pridham, Phytochemistry, 2 225 (1963).

10. Hydrolytic enzymes. J.B. Pridham, In "Enzyme Chemistry of phenolic compounds", (J.B. Pridham ed.) p.73. Pergamon Press, London, 1963.

11. The biosynthesis of phenolic glucosides in plants. J.B. Pridham and Margaret J. Saltmarsh, Biochem. J., 87, 218 (1963).

12. Deamination of β-(3,4-dihydroxyphenyl)-L-alanine by plants. Norma J. Macleod and J.B. Pridham, Biochem. J. 88, 45P (1963).

13. The phenol glucosylation reaction in the plant kingdom. J.B. Pridham, Phytochemistry, 3, 493 (1964).

14. Enzymatic galactosylation and fucosylation of phenols. J.B. Pridham and K. Wallenfels, Nature, 202, 488 (1964).

15. Paper electrophoresis of phenolic compounds. J.B. Pridham In "Methods in Polyphenol Chemistry", (J.B. Pridham ed.) p.111 Pergamon Press, London, 1964.

16. Oxidation and reduction of aromatic compounds by plants. J.B. Pridham and Margaret Young, Biochem J., 92, 20P (1964).

17. Phenol-carbohydrate derivatives in higher plants. J.B. Pridham, Advanc. Carbohydrate Chem., 20, 371, (1965).

18. Structure of a dopa glucoside from Vicia faba. R.S. Andrews and J.B. Pridham, Nature, 205, 1213 (1965).

19. Low molecular weight phenols in higher plants. J.B. Pridham, Ann. Rev. Plant Physiol., 16, 13 (1965).

20. Observations on the translocation of phenolic compounds. Norma J. Macleod and J.B. Pridham, Phytochemistry, 5, 777 (1966).

21. Melanins from DOPA-containing plants. R.S. Andrews and J.B. Pridham, Phytochemistry, 6, 13 (1967).

C. EDITED BOOKS SECTION*

1. "Phenolics in Plants in Health and Disease". J.B. Pridham ed. Pergamon Press, London, 1960.

2. "Enzyme Chemistry of Phenolic Compounds". J.B. Pridham ed. Pergamon Press, London, 1963.

3. "Methods in Polyphenol Chemistry". J.B. Pridham ed. Pergamon Press, London, 1964.

4. "Biosynthetic Pathways in Higher Plants". J.B. Pridham and T. Swain eds. Academic Press, London, 1965.

* One set only submitted.

CARBOHYDRATES

AND

NUCLEOTIDES

A1 - A23

A1

(*Reprinted from Nature, Vol.* 172, *p.* 207, *August* 1, 1953)

Free Xylose in Fruits

AN investigation of the carbohydrates present in the Victoria plum has revealed the presence of small amounts (*c.* 0·1 per cent fresh weight) of free xylose in the juice of ripe and immature fruits. The origin of this sugar is uncertain; it may arise from enzymic hydrolysis of a xyloside, but we consider this unlikely.

The xylose was isolated by maceration of the plum mesocarp, followed by dialysis against distilled water. The dialysate, containing the free sugars, was evaporated to a syrup *in vacuo*, and the xylose isolated by partition chromatography using a cellulose column[1].

The xylose was characterized by paper chromatography (Whatman No. 1 paper was employed) using as solvents ethyl acetate – acetic acid – water (9 : 2 : 2), *n*-butanol – pyridine – water (10 : 3 : 3), and phenol–water (phenol saturated with water). The chromatograms were developed by spraying with 4 per cent anisidine hydrochloride in *n*-butanol[2].

Further proof of identity was obtained by the preparation of a dimethyl acetal dibenzylidene derivative.

Sufficient material was not available for accurate optical measurements, though a preliminary examination showed that it exhibited a small dextrorotation.

Evidence has also been obtained of the existence of free xylose in other varieties of mature plum, damson and quince, and Dr. A. E. Flood and his colleagues have detected it chromatographically in young apple and pear shoots, and in the leaves of potato[3].

It is hoped to continue the investigation when further supplies of fruit are available. We are indebted to Dr. J. K. N. Jones for his advice and interest during this work.

J. G. HAY
J. B. PRIDHAM

Department of Biological Chemistry,
University, Bristol 8. March 26.

[1] Hough, L., Jones, J. K. N., and Wadman, W. H., *J. Chem. Soc.*, 2511 (1949).
[2] Hough, L., Jones, J. K. N., and Wadman, W. H., *J. Chem. Soc.*, 1702 (1950).
[3] Flood, A. E. (private communication).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

A2

[FROM THE BIOCHEMICAL JOURNAL, VOL. 58, No. 2, pp. 288–290, 1954]
 PRINTED IN GREAT BRITAIN

A Colorimetric Estimation of Sugars Using Benzidine

BY J. K. N. JONES* AND J. B. PRIDHAM
Department of Chemistry, The University, Bristol 8

(*Received* 1 *May* 1954)

Many colorimetric methods using organic reagents are available for the estimation of sugars, a number of which have been utilized in conjunction with chromatography. For example, a method using aniline phthalate has been described (Blass, Macheboeuf & Núnez, 1950; Bartlett, Hough & Jones, 1951). This method, however, consists of several stages, which is a disadvantage. Methods using concentrated mineral acids are to be avoided if possible, in order to simplify practical procedure, and for this reason the method described by Dubois, Gilles, Hamilton, Rebers & Smith (1951), using a phenol–sulphuric acid reagent, was considered unsuitable for routine analysis.

In connexion with work on gums, it was considered necessary to have available a simple colorimetric estimation applicable to all the monosaccharide constituents of complex polysaccharides and their methylated derivatives.

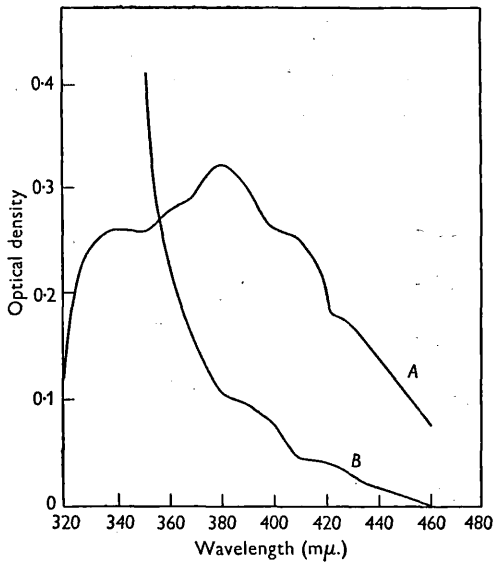


Fig. 1. Absorption spectra for: *A*, reaction of 100 μg. D-galactose with the benzidine reagent; *B*, the blank determination. Curves for aldopentoses, methyl aldopentoses, hexuronic acids, and methylated sugars were similar to *A*.

* Present address: Department of Chemistry, Queen's University, Kingston, Ontario, Canada.

Benzidine is used as a chromatographic spray reagent for reducing sugars (Horrocks & Manning, 1949) and for the estimation of pentoses by an indirect method involving furfural (McCance, 1926). An attempt was therefore made to estimate sugars directly using a solution of benzidine in glacial acetic acid. Preliminary experiments showed that aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and oligosaccharides all gave an orange-yellow coloration when heated with the reagent, and that the colour in each case had a similar maximum absorption band (Fig. 1). Further investigation showed that the relationship between absorption and concentration was linear in all cases studied.

It was observed that the benzidine reagent in glacial acetic acid gradually became pale yellow when exposed to air and light. However, the addition of a small amount of stannous chloride prevented this discoloration, and also increased the sensitivity, enabling the determination of much lower concentrations of sugar. Stannous chloride is also used in this laboratory to stabilize the *p*-anisidine hydrochloride spray reagent (Hough, Jones & Wadman, 1950).

The composition of polysaccharides was determined using the benzidine reagent, after hydrolysis and separation of the monosaccharide constituents on paper chromatograms.

EXPERIMENTAL

Reagent. This consists of a 0·2% (w/v) solution of benzidine (A.R.) in glacial acetic acid (A.R.) containing 0·1% (w/v) $SnCl_2$.

Owing to the slight instability of the reagent, the determination of sugars is not based on a standard curve, but estimations of standard sugar solutions are carried out together with the unknown. The solution of benzidine in glacial acetic acid should be freshly prepared. The addition of stannous chloride produces a cloudy precipitate which is filtered off. The clear, colourless solution is then ready for use.

Method

The estimations are carried out in test tubes (7 × 1 in.) provided with small glass spheres to prevent the entry or loss of water. Sugar solution to be determined (1 ml.), containing not more than 600 μg. of sugar, is added from a pipette into a tube, followed by the benzidine reagent (5 ml.) from a burette. After a thorough mixing, the tubes are heated in a vigorously boiling water bath for a period depending on the class of the sugar (Table 1 and Fig. 2). At

least two standards of suitable concentration are included and also a blank determination, i.e. 1 ml. of water and 5 ml. of benzidine reagent.

After being heated, the tubes are cooled in running water for not less than 3 min. The colour is then measured with a Hilger 'Spekker' photoelectric absorptiometer using a violet filter (Ilford no. 601, wavelength 425 mμ.) and a 0·5 cm. glass cell. The concentration of the unknown can then be calculated from the standards by simple proportion, or, better still, if three or more standards are employed, by plotting absorption against concentration and reading the unknown from the resulting graph. The relation between concentration and absorption was found to be linear over the range 100–600 μg./ml. for the sugars listed in Table 1.

Concentrations as low as 20 μg./ml. can also be determined satisfactorily, provided that standards are included within the range 20–100 μg./ml., and the calculation is based on these standards. The reason for this is that below a concentration of 100 μg./ml. the slope of the concentration/absorption graph changes slightly (Fig. 3). The method is not suitable for concentrations of less than 100 μg./ml. of methylated sugars and disaccharides or less than 50 μg./ml. of hexuronic acids. The determination may be carried out with errors of less than ±3%.

Table 1. *Times of heating sugars with the benzidine reagent*

Sugar	Heating time necessary for maximum colour production (min.)
Arabinose	15
Ribose	
Lyxose	
Xylose	
Glucose	30
Galactose	
Mannose	
Rhamnose	
Fucose	
Glucurone	
Galacturonic acid	
2:3:4-Trimethylxylose	60
2:4:6-Trimethylglucose	
2:3:6-Trimethylglucose	
2:3:4:6-Tetramethylglucose	
Maltose	
Sucrose	

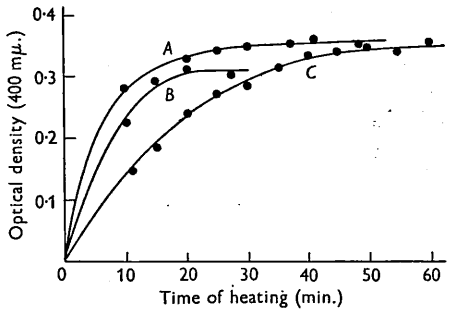


Fig. 2. Variation in optical density (400 mμ.) with time of heating for: *A*, D-glucurone (300 μg.); *B*, D-ribose (250 μg.); *C*, 2:4:6-trimethyl-D-glucose (160 μg.) with benzidine reagent. Methyl aldopentoses, aldohexoses, and galacturonic acid gave curves similar to *A*. All aldopentoses resembled curve *B* and all methylated sugars curve *C*. Measurements were made with a Unicam SP. 350 spectrophotometer using a 1 cm. glass cell.

The absorption spectra of the coloured compounds produced by interaction of the benzidine reagent with sugars were plotted at intervals of 5 mμ. using a Unicam S.P. 500 spectrophotometer, and a 1 cm. glass cell. The maximum absorption with all the sugars tested was in a wavelength band ranging from approximately 340 to 420 mμ. (Fig. 1). Small peaks were evident within the wavelength band, with a small variation depending on the nature of the sugar. The main absorption plateau, however, was of a similar nature in every case. The use of the 'Spekker' with a violet filter to measure the colour produced, instead of a spectrophotometer employing a diffraction grating for the selection of a definite wavelength, was found to be quite satisfactory. The former instrument was easier to operate, especially when dealing with many determinations, and the violet filter allowed absorption to be measured over a wavelength band where the absorption maximum of the blank determination was at a minimum.

Specificity of coloration

The benzidine reagent was observed to give colours with hexose diphosphate, 2-deoxy-D-galactose, dihydroxyacetone, and with ketonic compounds with free or easily accessible carbonyl groups, e.g. acetone, formaldehyde, α-oxoglutaric acid, etc. D-Fructose, however, only gave a colour when in high concentration.

No colour was observed with glycine (in concentration less than 1·0 mg./ml.), tryptophan, tyrosine, asparagine, cystine, hydroxyproline, urea, hexitols, or calcium gluconate. D-Glucosamine hydrochloride gave a faint colour in a concentration of 500 μg./ml.

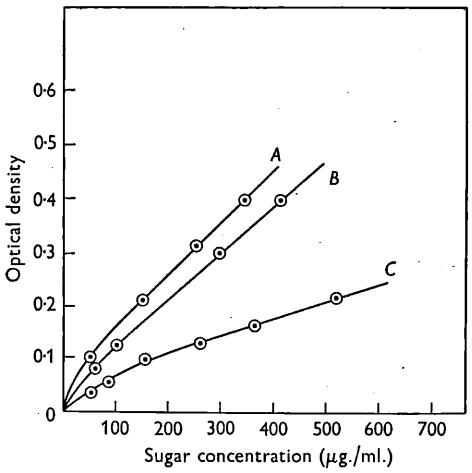


Fig. 3. Concentration/absorption graphs showing change of slope below a concentration of 100 μg./ml. for: *A*, D-mannose; *B*, D-xylose, *C*, D-glucurone. Other sugars show a similar change. The filter used (Ilford 601) transmits light in the range 385–470 mμ., max. transmission 425 mμ.

Table 2. *Analysis of polysaccharides*

Polysaccharide	Ratio of D-glucose or D-galactose to D-mannose: Determined by benzidine method	Ratio of D-glucose or D-galactose to D-mannose: Results of previous workers	Recovery based on ribose recovery (%)
Lucerne seed galactomannan	1:1·35	1:1·25*	101·8
Lilium umbellatum glucomannan	1:1·88	1:1·83†	94·4

* Andrews, Hough & Jones (1952).
† Andrews, Hough & Jones (unpublished results).

Table 3. *Recoveries of sugars after partition on paper*

Sugar	Amount added to paper (μg.)	Recovery (μg.)	Recovery (%)
L-Arabinose	309	309	100
	233	235	101
	155	165	106
D-Xylose	998	930	93
	750	750	100
	499	457	92
	250	229	92
D-Galactose	99	99	100
	398	365	92

Analysis of polysaccharides

Two polysaccharides have been hydrolysed, their constituent sugars separated on paper chromatograms (Flood, Hirst & Jones, 1948) and analysed using the benzidine reagent. The results obtained (Table 2) were comparable with those of other workers.

The sugars were eluted from the chromatograms by shaking the paper strips with suitable volumes of distilled water in tubes fitted with ground-glass stoppers. The sugar solutions were then poured off, centrifuged to remove small particles of cellulose, and samples taken for analysis (cf. Roudier, 1951).

The recovery from the paper using the above technique was examined by applying standard sugar solutions to chromatograms with an 'Agla' micrometer syringe (Burroughs Wellcome and Co., London), developing with ethyl acetate–acetic acid–water solvent (9:2:2, v/v) and eluting the sugar as described. The error was found to be within ±9% (Table 3).

Lucerne seed galactomannan. The polysaccharide (11·38 mg.) was hydrolysed with N-H_2SO_4 in a sealed tube overnight at 100°. D-Ribose (2·925 mg.) was added to the hydrolysate and then the acid neutralized with barium carbonate. After filtration, the solution was evaporated under reduced pressure to a small volume. The three component sugars were then separated chromatographically using *n*-butanol–ethanol–water solvent (40:11:19, v/v) (see Table 2).

Lilium umbellatum *glucomannan.* The glucomannan (20·27 mg.) was hydrolysed in a sealed tube with 98% formic acid at 100° overnight. The acid was removed by evaporation under reduced pressure, and the residue taken up in N-H_2SO_4. D-Ribose (6·318 mg.) was then added and the solution heated at 100° for 30 min. to hydrolyse formyl esters. The acid was then neutralized as before with barium carbonate, and the remainder of the procedure repeated as described for lucerne seed galactomannan (see Table 2).

SUMMARY

1. A new method of determining sugars colorimetrically is described, the reagent consisting of a solution of benzidine in glacial acetic acid. The method is suitable for the determination of aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and disaccharides. Ketoses and amino sugars cannot be estimated with this reagent.

2. From 20 to 600 μg./ml. of aldopentose, methyl aldopentose, and aldohexose may be determined by the method. Hexuronic acids can be estimated in concentrations ranging from 50 to 600 μg./ml. Methylated sugars and disaccharides cannot be estimated satisfactorily below a concentration of 100 μg./ml. The sugars may be determined with errors less than ± 3 %.

3. The analysis of a glucomannan and galactomannan has been carried out using this procedure.

One of us (J.B.P.) wishes to thank the Department of Scientific and Industrial Research (Food Investigation) for a grant.

REFERENCES

Andrews, P., Hough, L. & Jones, J. K. N. (1952). *J. Amer. Chem. Soc.* **74**, 4029.
Blass, J., Macheboeuf, M. & Núnez, G. (1950). *Bull. soc. chim. biol.* **32**, 130.
Bartlett, J., Hough, L. & Jones, J. K. N. (1951). *Chem. & Ind.* **4**, 76.
Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. & Smith, F. (1951). *Nature, Lond.*, **168**, 167.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1948). *J. Chem. Soc.* p. 1679.
Horrocks, R. H. & Manning, G. B. (1949). *Lancet*, **258**, 1042.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. Chem. Soc.* p. 1702.
McCance, R. A. (1926). *Biochem. J.* **20**, 1111.
Roudier, A. (1951). *Memorial des services chimiques de l'Etat*, **36**, 161. (Published by the Laboratoire Central des Services Chimiques de l'Etat, Paris.)

A3(a)

Abstracts. 128th American Chemical Society Meeting p. 17D, 1955

The formation of analogous compounds from D-arabo ascorbic acid and from D-gluco ascorbic acid is described.

35. THE COMPOSITION AND FUNCTION OF PLANT GUMS. L. Hough and J. B. Pridham, Biological Chemistry Laboratories, The University, Bristol, England.

Fruit surface gum and bark gum of Victoria plum are chiefly composed of polysaccharide materials which on acid hydrolysis yield D-glucuronic acid, D-galactose, D-mannose, L-arabinose, D-xylose, and L-rhamnose. The bark gum polysaccharide was shown to contain a higher proportion of D-mannose, L-arabinose, and hexuronic acid than the fruit surface gum.

Traces of combined 4-O-methylglucuronic acid were also detected in the polysaccharide of the fruit surface gum.

Periodate oxidation of the two types of gum also showed that they were structurally different. Both gum polysaccharides appeared to be electrophoretically homogeneous, and a mixture of the two could not be resolved.

Both types of Victoria plum gum contained peroxidase, as did samples of gums from several other plant species. The physiological significance of this enzyme in relation to the gum is discussed.

Also present in plum gums were phenolic materials, including lignin and small amounts of pentose- and hexose-containing oligosaccharides. The latter compounds could be extracted with cold methanol (pH 7), and therefore it is improbable that they arose by partial hydrolysis of the polysaccharides during experimentation. Oligosaccharides were also extracted from gum arabic and cherry gum with cold methanol.

A3(b)

Some Observations on the Composition, Function and Biogenesis of Plant Gums with Particular Reference to the Plum Gums.

By J. B. PRIDHAM (introduced by C. A. ASHFORD). (*The Biological Chemistry Laboratory, University of Bristol*)

Biochem. J. 57, xxviii (1954)

The gum that is exuded from the bark and fruit of plum trees, like other plant gums, consists largely of a complex acidic polysaccharide composed of D-glucuronic acid, D-galactose, D-mannose, L-arabinose, D-xylose and L-rhamnose (cf. Hirst & Jones, 1947).

It has been suggested (Hirst & Jones, 1951) that gummosis is a protective mechanism which enables the plant to seal off lesions made by mechanical injury or invading micro-organisms. Small-scale experiments indicate that gum formation is probably enhanced when micro-organisms are introduced artificially into plum trees, and repeated failures to degrade gums *in vitro* with micro-organisms illustrate the value of these compounds to the tree.

Apart from the large carbohydrate fraction, the exuded gums also contain small amounts of phenolic compounds, lignin and protein, and show peroxidase activity, as do gum arabic (Whistler & Smart, 1953), gum *Cedrela sinensis*, and grapefruit gum.

The proportions of D-galactose, D-mannose, L-arabinose and D-xylose, after hydrolysis, in Victoria plum bark and fruit gums, have been determined by the benzidine method (Jones & Pridham, 1953), and it has been shown that the bark gum contains a higher proportion of D-mannose and L-arabinose than the fruit gum. Other properties, however, such as optical rotation and rate of hydrolysis, are similar for both gums.

The use of the Tiselius electrophoresis apparatus for investigating the homogeneity of water-soluble polysaccharides has been explored, and artificial mixtures of gum arabic and plum gum in borate buffer (pH 7·8), and sugar-beet araban and plum gum in phosphate buffer (pH 7·0) have been sucessfully separated.

All attempts to fractionate bark and fruit gums electrophoretically, either singly or using an artificial mixture of the two, have so far failed.

The author wishes to thank the Department of Scientific and Industrial Research for a grant.

REFERENCES

Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1064.
Hirst, E. L. & Jones, J. K. N. (1951). *Research*, 4, 411.
Jones, J. K. N. & Pridham, J. B. (1953). *Nature, Lond.*, 172, 161.
Whistler, R. L. & Smart, C. L. (1953). *Polysaccharide Chemistry*, p. 310. New York: Academic Press.

A4

Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 59, No. 1, November, 1955
Academic Press Inc. *Printed in U.S.A.*

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 59, 17–23 (1955)

The Metabolism of D-Glucuronic Acid in Plum Tissue and Its Relation to Gummosis[1]

L. Hough and J. B. Pridham

From the Biological Chemistry Laboratories, The University, Bristol, England

Received May 2, 1955

INTRODUCTION

D-Glucuronic acid is a common constituent of polysaccharides, occurring in many plant gums and hemicelluloses (1). Experiments in this laboratory on the gummosis of plum trees have suggested that the formation of this uronic acid in the tree is related to the biosynthesis of plum gum. This gum is a complex acidic polysaccharide containing D-glucuronic acid, D-galactose, D-mannose, L-arabinose, D-xylose, and L-rhamnose (2). Degradation studies by other workers (3) on many plant gums have left little doubt that chemically the uronic acid occupies an important structural position in the gum molecule. It also probably helps to stabilize the molecule to microbiological attack, as all attempts to degrade plum gum with various microorganisms have failed (2), whereas these microorganisms hydrolyzed many other plant polysaccharides, e.g., pectic acid, araban, galactan, and xylan, thus supporting the suggestion that the gum functions as a protective barrier to microbiological invasion (4).

The formation and metabolism of D-glucuronic acid in animals has been studied extensively, and it now seems fairly certain that D-glucose is the precursor (5). However, few observations have been made in this connection using plant material. Isherwood, Chen and Mapson (6) studied the biosynthesis of ascorbic acid in cress seedlings and suggested that a possible route was the conversion of D-glucose to D-glucuronic acid.

Since the formation of gum by plum trees was accompanied by the appearance of combined glucuronic acid in the tissue polysaccharides, the effect of D-glucuronic acid and its lactone on the respiration of plum

[1] This work forms part of the program of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

tissues was studied. D-Xylose has been found in a free state in plum tissue (7), and therefore this experiment was of further interest owing to the suggestion that pentoses might arise by decarboxylation of hexuronic acids (8), since on stereochemical consideration alone, D-glucuronic acid is potentially a direct precursor of D-xylose.

The results obtained showed that sodium D-glucuronate was toxic, but that D-glucurono-γ-lactone was readily assimilated by plum shoots and produced a respiratory stimulation of plum fruit tissue slices. No evidence for the conversion of D-glucurono-γ-lactone to pentose by a decarboxylation mechanism was obtained. D-Xylose produced a slight inhibition of respiration of tissue slices.

Experimental

All experiments were carried out with organs or tissue preparations from trees of Victoria Plum; the tissue used for respiration measurements was collected in July.

Fresh plum fruit have been examined qualitatively for D-glucuronic acid at various stages of growth. The tissues were macerated, extracted with two portions of cold methanol, and after drying at room temperature, the tissue residue was hydrolyzed in *N* H_2SO_4 on a boiling water bath for approximately 16 hr. The mineral acid was then neutralized with barium carbonate (A.R.) and filtered, and the barium ions were removed from the filtrate by passing through a small column of Amberlite IR-120 resin (H^+ form). The eluate was then concentrated under reduced pressure and examined on paper chromatograms using ethyl acetate–acetic acid–water solvent (9:2:2 *v/v*) and Whatman No. 1 paper. The uronic acid, in the γ-lactone form, was detected by spraying with *p*-anisidine hydrochloride solution (9), when it appeared as a characteristic fast-moving pink spot, clearly distinguishable from the other monosaccharides present.

The effects of sodium D-glucuronate (0.5%) and D-glucurono-γ-lactone (1.0%) on plum fruit spurs were compared by immersing the cut ends into aqueous solutions of these compounds and leaving for approximately 24 hr. at room temperature. After this period, the general appearance of the spurs was noted, and the organs (leaves, fruit, and bast) were macerated separately, extracted with hot methanol, and, after evaporation of the methanol, the residue was examined on paper chromatograms for sugars.

Leaf respiration was measured manometrically by the method of Dixon (10), the petioles having been immersed in 1.0% solutions of D-glucose and D-glucurono-γ-lactone for 17 hr. at room temperature before the experiment. Gaseous exchange values were based on dry weight of leaves.

Respiration measurements on plum mesocarp tissues were made using the same manometric procedure. Cylindrical pieces (*ca.* 0.7 × 1.0 cm.) of tissue were cut with a No. 4 cork borer and partially immersed in water for 17 hr. prior to examination. Comparisons of respiration rates in water and 1.0% D-glucurono-γ-lactone solution, and in 1.0% D-glucose and 1.0% D-glucurono-γ-lactone solu-

tions were then carried out. The respiration of plum mesocarp tissue in water and in 1.0% D-xylose solution, without the preliminary starvation period in water, was also compared.

The possibility of decarboxylating D-glucuronic acid to form D-xylose was tested by incubating mesocarp slices with 1.0% D-glucurono-γ-lactone solution (10 ml.) for 4 days at room temperature. The supernatant liquor was then filtered through a weighed sintered-glass funnel (medium porosity), the tissue was extracted four times with boiling methanol to remove monosaccharides, and the residue was then transferred quantitatively to the funnel and washed with a further volume of methanol. The funnel and its contents were then dried to a constant weight in an oven at 100°. The combined washings were carefully evaporated to a sirup under reduced pressure, and diluted with water to 10 ml. D-Glucose and D-xylose were then separated chromatographically and estimated colorimetrically with benzidine using the method of Jones and Pridham (11). In a control experiment, the D-glucurono-γ-lactone solution was replaced with water (10 ml.) and analyzed simultaneously.

Results and Discussion

Qualitative paper chromatographic examination of acid hydrolysis products of plum mesocarp tissues at varying stages of growth indicates that galactose, mannose, arabinose, xylose, rhamnose, and galacturonic acid were present in the water-soluble polysaccharides of the tissues throughout the period of fruit development, and, apart from the galacturonic acid, in approximately the same proportions as in plum gum. Thus the mesocarp polysaccharides contain all of the monosaccharide constituents of the gum with the exception of glucuronic acid. The latter could not be detected in the mesocarp until late July, when external gummosis was evident from field observations, and then only in very low concentrations. Examination of the bast, however, by the same procedure, indicated that relatively high concentrations of the uronic acid were present in this tissue in June.

Qualitative observations with single plum fruit exhibiting external gummosis show that the glucuronic acid content of the mesocarp tissue adjacent to the gummosis site is much higher than that of tissue a short distance from the site.

These results suggest that the appearance of glucuronic acid in the mesocarp is directly related to the formation of gum. It seems plausible that the gum molecule is biosynthesized at a late stage in the development of the fruit, from pre-existing polymeric material such as the polysaccharides of the pectin complex or the cell wall hemicelluloses. In studies on the fixation of $C^{14}O_2$ by plum fruit spurs, it has been observed that

incorporation of C^{14} into pectin occurs[2] showing that this polysaccharide must be in a dynamic state during the period when fruit gummosis occurs, and could therefore act as a gum precursor. The incorporation of glucuronic acid into the gum might well be one of the final stages in the biosynthesis of this macromolecule. Small amounts of gum appear to be present in all plum fruit during the later stages of development, but the production of large amounts, both externally and internally, is probably due to some physiological disorder which at present is not understood.

The perfusion experiments showed that D-glucurono-γ-lactone was rapidly taken up by small plum fruit spurs, and chromatographic examination after 24 hr. showed that the lactone had been translocated to bast, leaves, and fruit. On replacing the spurs in water, they continued to appear normal for 2–3 days, after which brown lesions developed on the leaves followed by a loss of turgidity.

However, with sodium D-glucuronate, the leaves became pigmented and very brittle within a few hours, and no glucuronate uptake could be demonstrated.

Respiration measurements with plum leaves in D-glucurono-γ-lactone and D-glucose solutions showed that the R.Q. values were approximately unity in all cases, and no evidence was obtained for the existence of a system capable of decarboxylating the lactone to pentose. If decarboxylation had occurred, it is probable that the R.Q. value would have been greater when using the hexuronic acid or lactone as substrate than when using D-glucose.

Comparisons of the respiration of plum mesocarp tissue slices in water and D-glucurono-γ-lactone solution, and in D-glucose and D-glucurono-γ-lactone solutions (Tables I and II), suggest that the lactone was metabolized, as it stimulated the respiration of starved tissue slices, producing a 26% increase in O_2 consumption. However, the stimulation was more marked using D-glucose as substrate, as was previously found with plum leaves.

Again the R.Q. values obtained using D-glucurono-γ-lactone as substrate give no indication of decarboxylation, but in all cases the CO_2 output was much higher than the O_2 uptake. This is not unusual in succulent tissues and may be due to fermentation reactions under conditions of oxygen limitation.

Quantitative paper chromatography of an extract of mesocarp tissue, after it had been incubated with D-glucurono-γ-lactone solution, showed

[2] L. Hough and J. B. Pridham, unpublished results.

TABLE I

Respiration of Plum Mesocarp Tissue in Water and in 1% D-Glucurono-γ-lactone Solution

		Microliters gas/g. dry wt.		
		CO_2	O_2	R.Q. values
Water	1	445	221	2.0
	2	439	209	2.1
	3	367	218	1.7
Average value		417	216	
D-Glucurono-γ-lactone	1	521	266	2.0
	2	523	247	2.1
	3	638	381	1.7
Average value		561	298	

TABLE II

Respiration of Plum Mesocarp Tissue in 1% Solutions of D-Glucose and D-Glucurono-γ-lactone

		Microliters gas/g. dry wt.		
		CO_2	O_2	R.Q. values
D-Glucose	1	206	115	1.8
	2	254	125	2.0
	3	392	246	1.6
Average value		284	162	
D-Glucurono-γ-lactone	1	189	118	1.6
	2	238	133	1.8
	3	306	215	1.4
Average value		244	155	

TABLE III

Estimation of Glucose and Xylose in Plum Mesocarp Tissue after Incubation with Water and 1% D-Glucurono-γ-lactone Solution

	Per cent sugars (based on dry weight of tissue)	
	Glucose	Xylose
Tissue + D-glucurono-γ-lactone	28.3	6.2
Tissue + water	26.6	6.4

no significant change in the concentrations of glucose or xylose (Table III).

D-Xylose was found to produce no stimulation of fruit mesocarp respiration; instead a slight inhibition (16% decrease in O_2 uptake) was observed (Table IV).

While there is little doubt that D-glucurono-γ-lactone was metabolized by plum tissue, a process involving decarboxylation of the hexuronic

TABLE IV

Respiration of Plum Mesocarp Tissue in Water and in 1% D-Xylose Solution

		Microliters gas/g. dry wt.		
		CO_2	O_2	R.Q. values
Water	1	317	196	1.6
	2	330	195	1.7
	3	298	175	1.7
Average value		315	189	
D-Xylose	1	296	170	1.7
	2	309	147	2.1
Average value		302	159	

acid to D-xylose is improbable. Therefore it seems probable that the lactone was metabolized by the plant after initial conversion to D-glucose, which is in accord with observations of Isherwood, Chen, and Mapson (6). Furthermore, *Escherichia coli* adapted to growth on either D-galacturonic or D-glucuronic acids would not metabolize L-arabinose and D-xylose respectively, again suggesting that the uronic acids were not metabolized by initial decarboxylation (12).

The path of conversion of D-glucuronic acid to D-glucose is at present unknown, although the interconversion of these compounds may take place under the influence of uridine diphosphate (UDP), as UDP-glucose (13) and UDP-glucuronic acid (14, 15), since both have been isolated from living organisms. The reverse process, D-glucose to D-glucuronic acid, is better understood and proceeds directly without fragmentation in animals (5) and bacteria (16). It has recently been shown that a particle-free supernatant fluid from liver homogenates oxidized UDP-glucose to UDP-glucuronic acid in the presence of DPN^+ (15).

Acknowledgments

We thank Dr. E. C. Bate-Smith and Dr. B. Folkes for their helpful advice.

Summary

1. The appearance of combined glucuronic acid in plum mesocarp tissues coincided with the formation of fruit gum.

2. D-Glucurono-γ-lactone was metabolized by plum mesocarp tissues. No evidence was obtained for the conversion of the lactone to D-xylose by a decarboxylation process.

References

1. Whistler, R. L., and Smart, C. L., "Polysaccharide Chemistry." Academic Press, New York, 1953.

2. PRIDHAM, J. B., *Biochem. J.* **53**, xxviii (1954).
3. HIRST, E. L., AND JONES, J. K. N., *Research* (London) **4**, 411 (1951).
4. SWARBRICK, J., *J. Pomol. Hort. Sci.* **6**, 137 (1927).
5. DOUGLAS, C. F. J., AND KING, C. G., *J. Biol. Chem.* **202**, 865 (1953); ROSEMAN, A., LUDOWIEG, J., MOSES, F. E., AND DORFMAN, A., *J. Biol. Chem.* **206**, 665 (1954); BIDDER, T. G., *J. Am. Chem. Soc.* **74**, 1616 (1952); EISENBERG, F., AND GURIN, S., *J. Biol. Chem.* **195**, 317 (1952).
6. ISHERWOOD, F. A., CHEN, Y. T., AND MAPSON, L. W., *Biochem. J.* **56**, 15, 21 (1954).
7. HAY, J. G., AND PRIDHAM, J. B., *Nature* **172**, 207 (1953).
8. HIRST, E. L., *J. Chem. Soc.* **1949**, 522.
9. HOUGH, L., JONES, J. K. N., AND WADMAN, W. H., *J. Chem. Soc.* **1950**, 1702.
10. DIXON, M., "Manometric Methods." University Press, Cambridge, 1951.
11. JONES, J. K. N., AND PRIDHAM, J. B., *Biochem. J.* **58**, 288 (1954).
12. COHEN, S. S., *J. Biol. Chem.* **177**, 607 (1949).
13. LELOIR, L. F., *Advances in Enzymol.* **14**, 193 (1953).
14. DUTTON, G. J., AND STOREY, I. D. E., *Biochem. J.* **59**, 279 (1955).
15. STROMINGER, J. L., KALCKAR, H. M., AXELROD, J., AND MAXWELL, E. S., *J. Am. Chem. Soc.* **76**, 6411 (1954).
16. ROSEMAN, S., MOSES, F. E., LUDOWIEG, J., AND DORFMAN, A., *J. Biol. Chem.* **203**, 213 (1953).

A5

(*Reprinted from Nature, Vol.* 177, *p.* 1039 *only, June* 2, 1956)

Incorporation of Carbon-14 into the Complex Polysaccharides of Plants

THE carbon-14 tracer technique would appear to offer a unique opportunity for investigations of the biosynthesis and subsequent metabolism of pectins and hemicelluloses, provided that there is a measurable synthesis and turnover of these polysaccharides in plant tissues. After allowing four mature fruiting spurs, each with four to six leaves and two fruits, detached from plum trees (variety Victoria), to photosynthesize[1] in the presence of carbon-14 dioxide (from 43·5 μc. barium carbonate-^{14}C), the leaf polysaccharides and the water-soluble polysaccharides of the fruit mesocarp were isolated. Labelling of all the monosaccharide constituents was observed by hydrolysing the polysaccharides (*N* sulphuric acid; 100°; 16 hr.), separating the products on paper chromatograms and examining with a Geiger counter those areas containing the separated sugars. The specific activities of the latter were determined, after elution from the paper chromatograms, as infinitely thin films on planchets (see Table 1). The incorporation of carbon-14 into the fruit mesocarp polysaccharides may be due to photosynthesis by the fruit or by translocation from the leaves.

Table 1

Monosaccharide	Specific activity (counts/min./mgm.)	
	Leaf polysaccharides	Mesocarp polysaccharides
Galacturonic acid	882	20
Galactose	1,342	31
Glucose	3,110	—
Arabinose	334	11
Xylose	180	—
Rhamnose	184	—

The highly active glucose undoubtedly arose from leaf starch. Clearly, the complex polysaccharides are biosynthesized in the leaf and mature plum fruit tissues, thus suggesting the existence of a dynamic equilibrium between these polysaccharides and their monosaccharide constituents in a manner analogous to the animal proteins. Jermyn and Isherwood[2] have made an analytical study of the cell-wall polysaccharides of pear fruit at various stages of ripening, with the conclusion that certain of the polysaccharides

were being synthesized in over-ripe fruit. The synthesis of complex polysaccharides in mature fruit tissues is contrary to the classical view.

This work forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

L. HOUGH
J. B. PRIDHAM*

Department of Organic Chemistry,
University,
Bristol 8.

* Present address: The Institute of Paper Chemistry, Appleton, Wisconsin, U.S.A.

[1] Livingstone, L. G., and Medes, G., *J. Gen. Physiol.*, **31**, 75 (1947).
[2] Jermyn, M. A., and Isherwood, F. A. (unpublished results). Jermyn, M. A., Ph.D. thesis, University of Cambridge (1949).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Reprinted from ANALYTICAL CHEMISTRY, Vol. 28, Page 1967, December 1956

Determination of Sugars on Paper Chromatograms with *p*-Anisidine Hydrochloride

JOHN B. PRIDHAM[1]

The Institute of Paper Chemistry, Appleton, Wis.

A new method for the determination of aldopentoses, aldohexoses, hexuronic acids, and 6-deoxyaldohexoses involves the paper chromatographic resolution of the sugar mixture, spraying of the chromatogram with *p*-anisidine hydrochloride reagent, and heating the paper in an oven. The resulting colored spots are cut out, the colors eluted from the paper with aqueous methanol containing stannous chloride, and then the absorbance is measured with a spectrophotometer. The absorbances of the sugar–*p*-anisidine complexes are directly proportional to the weights of the sugars over a range of 5 to 50 γ. The method has been used as a rapid analytical tool for the determination of the monosaccharide components of wood pulps and hemicelluloses.

PAPER partition chromatography has proved to be an invaluable aid to the quantitative determination of the components of complex mixtures of sugars (*1*). However, the chromatographic procedure which is normally used has the following disadvantages: Relatively large amounts of the sugars must be applied to the paper; the location of the bands by the use of marker strips may be inaccurate; and the complete elution of the sugars is time-consuming.

In the procedure described here, the solution to be analyzed is applied to the paper as small discrete spots, thus ensuring good chromatographic separation when only a relatively short solvent development time is used. After development the chromatogram is dried, then sprayed with *p*-anisidine hydrochloride (*2*) reagent and heated. The resulting colored spots, the boundaries of which are clearly discernible, are then cut from the chromatogram. The colors can be rapidly eluted and measured in a spectrophotometer. The intensity of color bears a linear relationship to the amount of sugar over the range from 5 to 50 γ.

The method has been successfully used for the determination of aldohexoses, aldopentoses, hexuronic acids and 6-deoxyaldohexoses, and, therefore, has been particularly useful for the analysis of wood pulps and hemicelluloses.

REAGENTS

***p*-Anisidine Hydrochloride Solution.** Dissolve 1.0 gram of the crystalline reagent in 10 ml. of absolute methanol and make up to 100 ml. with 1-butanol. Add 0.1 gram of sodium hydrosulfite. Store the solution in the refrigerator.

Eluent. Dissolve 1.0 gram of stannous chloride in 5 ml. of water. Add 90 ml. of absolute methanol and filter the solution.

PROCEDURE

Quantitative analysis of sugar solutions was carried out by spotting suitable volumes onto Whatman No. 1 filter paper with an ultramicroburet. Three or four spots of standard mixed sugar solutions, the components of which corresponded qualitatively to those in the unknown samples, were also applied to the chromatograms. The standard solutions were spotted so that each sugar component was present in varying amounts over a range of 5 to 50 γ.

The chromatograms were developed with 1-butanol–pyridine–water solvent (6 to 4 to 3 v./v.) or ethyl acetate–acetic acid–water (9 to 2 to 2 v./v.) solvent for a minimum of 20 hours. The chromatograms were air dried, then sprayed as uniformly as possible with the *p*-anisidine hydrochloride reagent. They were then heated in an oven at 130° C. for 10 minutes. The colored sugar spots, together with suitable blanks, were cut out with a scissors, the areas of the pieces of paper being kept constant for each sugar series and as small as possible.

The color was eluted from the paper by shaking mechanically for 5 minutes with 3 ml. of methanol–stannous chloride solution in rubber-stoppered test tubes, 6 × 3/4 inch. The blanks were eluted similarly.

The absorbances were then measured in 1-cm. cells in a Beckman Model DU spectrophotometer at the following wave lengths:

Aldopentoses and hexuronic acids	510 mμ
Aldohexoses	400 mμ
6-Deoxyaldohexoses	385 mμ

Absorbance measurements on aldopentoses or hexuronic acids were carried out within a period of 30 minutes after development of the color.

Absorbance values were plotted against the weights of sugars for the standard determinations and the resulting graphs then used to determine the unknowns.

RESULTS AND DISCUSSION

An examination of the absorption spectra of the colored compounds formed by the interaction of sugars with the *p*-anisidine hydrochloride reagent showed that both aldopentoses and hexuronic acids gave compounds which had absorption maxima at 510 mμ. This suggests that the same reaction mechanism occurs with both types of sugar, possibly an initial degradation to furfural followed by a condensation of the furfural with the *p*-anisidine to form a compound with a characteristic red color. Small

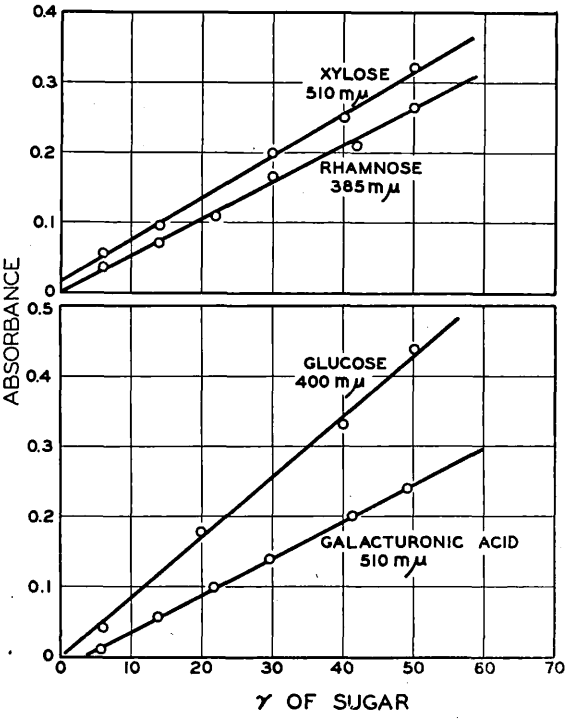


Figure 1. Increase in absorbance with increase in weight of various sugars

[1] Present address, Department of Chemistry, University of Edinburgh, Edinburgh 9, Scotland.

Table I. Quantitative Analysis of Spruce Pulp Hydrolyzed with 72% Sulfuric Acid

Sugar	Volume of Hydrolyzate Applied to Chromatogram, μl.	Determined Weight of Sugar, γ	Calcd. Sugar Concn. in Hydrolyzate, Mg./Ml.
Glucose[a]	5	12.2	24.4
	10	24.9	24.9
Mannose[b]	20	28.3	1.30
	30	38.2	1.40
Xylose[b]	40	39.0	0.97
	60	55.0	0.92

Sum of av. of sugar in hydrolyzate, 27.0 mg./ml.

[a] Hydrolyzate diluted 10 times; calculated sugar values corrected or dilution.
[b] Hydrolyzate undiluted.

variations in the shade of this color for different sugars, however, indicate that the reaction mechanism may be somewhat more complex than this.

The absorption maxima of the brown-colored compounds formed with aldohexoses and 6-deoxyaldohexoses were at 400 mμ and 385 mμ, respectively. The colors obtained with these sugars were relatively stable, but for best results, the absorbance should be measured shortly after color development.

The compound produced by reaction of aldopentoses or hexuronic acids with the *p*-anisidine hydrochloride reagent was rather unstable, but the addition of stannous chloride to the eluting solution enhanced the color stability. Nevertheless, for a pentose determination, it is advisable to measure the absorbance of the solution within a period of 30 minutes after the development of the color on the chromatogram.

The determination of standard sugars together with the unknowns on the same paper chromatogram has alleviated the use of exactly standardized conditions, so that small variations in the procedure given will not affect the final results. Some typical examples of curves obtained with standard sugars are shown in Figure 1. The errors are within ±4%.

Table I shows data obtained for the quantitative analysis of spruce pulp hydrolyzed with 72% sulfuric acid, using the procedure developed by Saeman and coworkers (*3*). In order to determine glucose, which was present in a high concentration in the pulp, the hydrolyzate was diluted 10 times before it was applied to the chromatogram. Mannose and xylose determinations were made using undiluted hydrolyzate.

A determination of the total sugar present in the hydrolyzate was carried out by cautious evaporation of a known volume at 50° C. under reduced pressure to a constant weight sirup. This gave a value of 27.3 mg. per ml. On this basis the figure given for the total sugar concentration in Table I represents a 99% recovery.

ACKNOWLEDGMENT

The author wishes to thank Maija Lakstigala for her interest and criticism.

LITERATURE CITED

(1) Hough, L., in "Methods of Biochemical Analysis," Vol. I, D. Glick, ed., p. 205–42, Interscience, New York, 1954.
(2) Hough, L., Jones, J. K. N., Wadman, W. H., *J. Chem. Soc.* **1950**, 1702.
(3) Saeman, J. F., Moore, W. E., Mitchell, R. L., Millett, M. A., *Tappi* **37**, 336–43 (1954).

RECEIVED for review April 7, 1956. Accepted August 16, 1956.

PRINTED IN U. S. A.

Reprinted from Chemistry and Industry, August 31, 1957, pp. 1178—1179

EFFECT OF ACID CONCENTRATION AND TEMPERATURE ON THE REVERSION OF L-ARABINOSE

*By L. Hough and J. B. Pridham**

Department of Chemistry, The University, Bristol

During studies of the reaction of L-arabinose with hydrogen peroxide solutions, it was observed that concentration of the reaction mixtures over a boiling water bath produced oligosaccharides. The cause of this reversion process was traced to the slight acidity of the solutions during evaporation. In view of the importance of selective acid hydrolysis, followed by characterization of the resultant oligosaccharides, in the structural analysis of polysaccharides, the reversion of L-arabinose has been examined at different temperatures and acid concentrations.

Solutions of L-arabinose (1% w/v) in 0·01N, 0·1N, 1·0N, 2·0N, 4·5N, 9·0N, and 18N sulphuric acid were kept for three days at 37°. After careful neutralization to pH 7·0 with either Amberlite resin IR-4B (OH^- form) or barium carbonate (A.R.), the filtered solutions were evaporated under reduced pressure and examined on paper chromatograms. With acid concentrations of 2N and below, no reversion products were detected, but with 4·5N, 9·0N, and 18N acid concentrations, two oligosaccharide spots (R_{GAL} 0·16 and 0·70)† were observed. The latter was indistinguishable from an authentic specimen of 3-*O*-β-L-arabopyranosyl-L-arabinose which has been detected along with the 4-*O*-isomer and *O*-β-L-arabopyranosyl-β-L-arabinoside in the reversion products of 6N hydrochloric acid on L-arabinose.[1] At 100° both products were relatively stable to 0·01N sulphuric acid, but completely hydrolysed to arabinose with N sulphuric acid indicating that they were arabopyranosyl derivatives. Epimeric products, namely ribose and *erythro*pentulose, were detected in the 4·5N- and 9·0N-acid-arabinose mixtures.

The effect of concentrating solutions of L-arabinose (1% w/v) in very dilute sulphuric acid (5 ml.) under reduced pressure over a boiling water bath was studied. 0·01N- and 0·001N-Acid solutions gave at least six reversion products moving more slowly than L-arabinose on paper chromatograms.† Similar treatment of 0·001N sulphuric acid solutions of D-glucose, D-galactose, D-mannose, D-xylose, D-ribose, and L-rhamnose gave rise to reversion products. Evaporation of a solution of L-arabinose in 0·0001N-acid produced epimers,[2] but no oligosaccharides.

In order to examine the effect of temperature on the reversion of solutions of L-arabinose (3% w/v) in 0·01N sulphuric acid (2 ml.), evaporation under reduced pressure was carried out at 40°, 60°, 80° and 100°, the evaporation time being kept constant (10 min.). Each syrup was then dissolved in water (3 ml.), neutralized with Amberlite IR-4B (OH^-) and equal volumes (0·02 ml.) subjected to paper chromatography.† In this way it was shown that oligosaccharides were formed at 60°, 80°, and 100° and that reversion was more extensive at 60°. At 100°, only traces of the lower molecular weight oligosaccharides remained, whereas at 60–80° they were preponderant. No reversion products could be detected in the solution evaporated at 40°, thus emphasizing the point that solutions of reducing sugars should never be evaporated at temperatures $> 40°$. Care should also be taken to exclude all traces of acid when concentrating sugar solutions, particularly at elevated temperatures.

The preparation of oligosaccharides by reversion would appear to be facilitated by evaporating dilute acid solutions at elevated temperatures rather than by leaving for prolonged periods in strong acid.[1,3–6]

Received July 26, 1957

* Present address: Royal Holloway College, Englefield Green, Surrey

† Solvent: ethyl acetate—acetic acid—water (9:2:2 v/v). Spray reagent: *p*-anisidine hydrochloride. The spots are probably mixtures of closely related oligosaccharides.

References

1 Ball, Jones, Nicholson & Painter, *Tappi*, 1956, **39**, 438; Jones & Nicholson, *J. chem. Soc.*, forthcoming publication

2 *cf.* Hassid, Doudoroff, Barker & Dore, *J. Amer. chem. Soc.*, 1946, **68**, 1465

3 Myrback, Hammèrstrand & Gelinder, *Arkiv. Kemi*, 1949, **1**, 235

4 Bacon & Bacon, *Biochem. J.*, 1954, **58**, 396

5 Turton, Bebbington, Dixon & Pacsu, *J. Amer. chem. Soc.*, 1955, **77**, 2565

6 Fetzer, Crosby, Engel & Kirst, *Ind. Eng. Chem.*, 1953, **45**, 1075

PRINTED BY RICHARD MADLEY LTD., 54 GRAFTON WAY, LONDON, W.1, ENGLAND

A8

[FROM THE BIOCHEMICAL JOURNAL, VOL. 73, No. 3, pp. 550–559, 1959]
 PRINTED IN GREAT BRITAIN

The Composition of Plum Gums

BY L. HOUGH
Chemistry Department, University of Bristol

AND J. B. PRIDHAM
Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 1 *May* 1959)

Gummosis, or the formation of gums, is a common phenomenon in the plant kingdom, and in temperate climates fruit trees of the Rosaceae family, e.g. peach, plum, almond, etc., are particularly susceptible. These gums may appear as exudates on the epidermal surfaces of the various plant organs or as internal pockets ('stone gum') within the tissues. They are composed predominantly of carbohydrate in the form of inorganic salts of complex acidic polysaccharides.

The biosynthetic mechanisms involved in the formation of gum polysaccharides are not understood. It has been variously suggested that gums are the products of micro-organisms (Grieg-Smith, 1904; Butler & Jones, 1949) and that they are produced from pectin (Higgins, 1919) and from hemicellulose (Sands & Gary, 1933). Recent histochemical studies by Ceruti & Scurti (1954) have shown that the precursor of cherry gum (*Prunus avium* L.) is probably a galactan. There seems little doubt that the gums originating from *Prunus* species are metabolic products of the plant normally found in the tissues but produced in excess when the plant is stimulated by mechanical or microbiological injury (Hirst, 1949) or by some physiological disorder. The apparent function of gum formation is to seal off surface wounds and to prevent the spread of invading micro-organisms.

This paper describes some studies on the composition of plum gums in relation to their possible function in the plant. A preliminary account of this work has been given by Pridham (1954).

MATERIALS AND METHODS

General methods. M.p. values are uncorrected. Optical rotations are given as equilibrium values in aqueous solution at 25°. Evaporations were carried out under reduced pressure. A Unicam SP. 500 spectrophotometer (1 cm. cell) was used for all u.v. measurements.

Purification of the gum polysaccharides. The gums, which were collected from trees (var. Victoria) were vigorously shaken with water at room temperature, the solutions were clarified in the centrifuge and the polysaccharides precipitated from the supernatant liquors by addition of excess of ethanol. After isolation by centrifuging, the white precipitates were washed with absolute ethanol followed by ether, and dried at 60° under reduced pressure.

Hydrolysis of the gum polysaccharides. (i) Qualitative. The gums were hydrolysed in N-H_2SO_4 in sealed glass tubes for 16 hr. After neutralization ($BaCO_3$) and concentration, the residues were examined on paper chromatograms. Barium ions were removed from the hydrolysates by treatment with Amberlite IR-120 (H) resin and, after concentration, the residues were again examined on paper chromatograms for glucurono-γ-lactone, which appeared as a characteristic red spot [$R_{galactose}$ 4·8 in solvent (1), see below] with the *p*-anisidine hydrochloride reagent. Further characterization of the monosaccharide constituents of the fruit-surface gum was carried out as follows. The gum (5 g.) was hydrolysed in N-H_2SO_4 (160 ml.) at 95–100° for 21 hr. After neutralization ($BaCO_3$) the solution was evaporated to a thick syrup which was dried by repeated evaporation in the presence of methanol. The resulting material was then extracted with boiling methanol (100 ml.; three portions) to separate the neutral monosaccharides (*A*) from the insoluble barium salts (*B*). Evaporation of the filtered methanol extracts gave a mixture of sugars (*A*; 2·54 g.) which was fractionated by chromatography on a column of cellulose with aq. butan-1-ol (half-saturated with water) as the mobile phase. The barium salts (*B*, 4·46 g.) were dissolved in water and then passed through a column of Amberlite IR-120 (H) resin, and the acidic effluent was concentrated to a small volume. After examination on paper chromatograms this solution was passed through a charcoal–Celite column, which was washed with water to elute D-glucurono-γ-lactone. Further elution of the column with aq. 50% ethanol afforded a mixture of oligosaccharides (*C*, 139 mg.). A portion (39 mg.) of this mixture (*C*) was hydrolysed (2N-H_2SO_4, 100°, 16 hr.) and the neutralized ($BaCO_3$) hydrolysate examined on paper chromatograms. The remainder of this mixture (*C*, 100 mg.) was heated under reflux with methanolic 4% hydrogen chloride until the solution was non-reducing to Fehling's solution. The neutralized (Ag_2CO_3) solution was concentrated, the resulting syrup was dissolved in tetrahydrofuran and treated with lithium aluminium hydride (Lythgoe & Trippett, 1950). Excess of hydride was decomposed with ethyl acetate, the solution was poured into ice–water and then filtered. Concentration gave a mixture of methyl glycosides which were hydrolysed (N-H_2SO_4, 100°, 16 hr.) and then examined on paper chromatograms.

(ii) Quantitative. (*a*) The fruit-surface and bark gums were hydrolysed in N-H_2SO_4 by heating in an autoclave (15 lb./in.^{2}, 120°) for exactly 2 hr. The hydrolysates were neutralized with Amberlite IR-4B (OH) and the sugars determined by the benzidine method (Jones & Pridham, 1954). (*b*) The analyses were repeated, as described above, but the period of hydrolysis was reduced to 1 hr. with N-H_2SO_4 at 15 lb./in.^{2} pressure.

Paper chromatography. All separations were carried out on Whatman no. 1 paper by the descending method, unless otherwise stated, with one of the following solvent systems: (1) ethyl acetate–acetic acid–water (9:2:2, by vol.); (2) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.); (3) butan-1-ol–pyridine–water (10:3:3, by vol.); (4) butan-1-ol–ethanol–water (40:11:19, by vol.); (5) phenol–water (bottom layer); (6) butan-1-ol–acetic acid–water (2:1:1, by vol.); (7) propan-1-ol–water (4:1, v/v); (8) light petroleum (b.p. 100–110°) saturated with water. Solvents (5) and (6) were used for the examination of amino acids which were detected by the use of ninhydrin.

Reducing sugars were located on the paper chromatograms with the following spray reagents: (*a*) ammoniacal $AgNO_3$ (Partridge, 1948); (*b*) *p*-anisidine hydrochloride; (*c*) resorcinol–HCl (Hough, Jones & Wadman, 1950).

Phenols and their derivatives were separated by using solvents (4) and (6) and they were located on the paper chromatograms with diazotized *p*-nitroaniline–Na_2CO_3 (Swain, 1953) and diazotized sulphanilic acid (Evans, Parr & Evans, 1949) spray reagents. In some cases the spots were also detected by their fluorescence under ultraviolet light with or without exposure to NH_3.

Phosphates were examined on Whatman no. 542 acid-washed paper, irrigated with solvent (2) and subsequently detected by the procedure of Hanes & Isherwood (1949).

Alkaline nitrobenzene oxidation products were detected on paper chromatograms with 2:4-dinitrophenylhydrazine solution (0·3% in 2N-HCl, Stone & Blundell, 1951).

The inorganic cations present in fruit-surface gum and stone gum of plums were studied by the qualitative paper chromatographic procedures described by Pollard & McOmie (1953).

Hexuronic acid anhydride content. The method of Johannson, Lindberg & Theander (1954) was used, and each sample of gum (approx. 0·1 g.) analysed in duplicate. The results were corrected for ash and water content of the gums.

Periodate oxidations. A known weight of the gum was dissolved in water by vigorous agitation at room temperature; 0·3 M-sodium metaperiodate solution (20 ml.) was added and the volume made to 100 ml. with water. A control solution containing 0·3 M-sodium metaperiodate solution (20 ml.) and water (80 ml.) was prepared at the same time. The oxidation was carried out in the dark and at room temperature. At varying intervals of time, two samples (5 ml. each) were withdrawn from each flask. To one sample ethylene glycol (2 ml.) was added; it was then diluted to about 12 ml. with water and the acidity titrated potentiometrically with 0·01 N-NaOH (Anderson, Greenwood & Hirst, 1955). The other sample was added to a mixture of 20% KI solution (5 ml.) and 2N-H_2SO_4 (5 ml.) and the periodate content was determined by titration of the liberated iodine with 0·1 N-sodium thiosulphate (Schwartz, 1954). Oxidations were carried out on crude and purified gums.

Electrophoresis. The gum was dissolved in aqueous buffer solution by vigorous agitation at room temperature. After centrifuging, the solution was dialysed against buffer until the pH and conductivity of both buffered gum solution and the dialysate were approximately the same. Electrophoresis was carried out at 4° in a Hilger electrophoresis apparatus at a potential of 280–380 V and a current of 12–18 MA.

Mono- and oligo-saccharides. Crude gums were finely powdered and extracted by shaking with methanol at room temperature for 20 hr. The filtered extracts were concentrated and examined on paper chromatograms. In one instance extraction was effected with methanol containing about 1% of aq. NH_3 soln.

Lignin. Crude gum was heated with 2N-H_2SO_4 at 100° for 16 hr., care being taken to see that the samples did not contain any plant tissue. The residue was then filtered off and subjected to alkaline nitrobenzene oxidation (Freudenberg, Lautsch & Engler, 1940). The resulting products were examined on paper with solvent (8) and 2:4-dinitrophenylhydrazine as spray reagent.

Phenolic compounds. The gum was agitated with an excess of water at room temperature, the solution was clarified by centrifuging, acidified with acetic acid and then extracted continuously with ether for 10 hr. After concentration, the ethereal extract was examined on paper chromatograms. Further extraction of the aqueous gum solution was then carried out with ethyl acetate: this extract and the residual aqueous solution were concentrated and the syrups examined on paper chromatograms.

Amino acids and protein. Several specimens of gum were analysed for nitrogen. Gums were examined by paper chromatography, before and after partial hydrolysis, for ninhydrin-positive compounds. Partial hydrolysis was effected by leaving a 1% solution of gum in 3N-HCl at room temperature for 3 weeks. The acid was neutralized (Ag_2CO_3) and the solution then treated with H_2S and concentrated.

Peroxidase. Benzidine (Madeling, 1911) and pyrogallol (Willstätter & Stoll, 1918), both in the presence of traces of H_2O_2, were used as colorimetric spot tests for peroxidase.

Peroxidase activity was measured by adding a saturated aqueous solution of benzidine (0·04%; 5 ml.) to a gum solution diluted to 25 ml. Hydrogen peroxide (20 vol.; 0·05 ml.) was then added and after 5 min. the resulting blue was measured with an EEL colorimeter (Ilford no. 608 filter, peak transmission 680 mμ) with diluted gum solution as a blank.

Determination of reducing substances in plum-fruit tissues. Fruits were selected in July and cylindrical pieces of tissue removed from gumming and non-gumming mesocarps with a cork borer (no. 4). The tissue was disintegrated with a spatula and individually extracted three times with small volumes (10 ml.) of boiling methanol. Each extract was poured through a weighed sintered-glass funnel (medium porosity) and, finally, the tissue residues were transferred quantitatively to the funnels, washed with hot methanol (20 ml.) and dried to constant weights at 100°. The methanolic extracts were concentrated to syrups, then diluted with water to 10 ml. and small portions were analysed for reducing sugars by the Somogyi (1945) and benzidine methods (Jones & Pridham, 1954). Examination of these extracts on paper chromatograms showed that the main reducing component was glucose. The extracts were then separated on paper chromatograms and the glucose was determined with benzidine.

Respiration of plum-fruit tissues. Cylinders (1 cm. × 1 cm.) of mesocarp tissue were cut from healthy plum fruit and from gumming fruit (tissues adjacent to the 'lesion') with a cork borer. Respiration measurements were by the manometric method of Dixon (1951).

Examination of plum-fruit tissues for micro-organisms. Gumming fruits were surface-sterilized with ethanol, the nodules carefully removed and pieces of tissue from beneath were plated on to a nutrient-agar medium [Lab Lemco (10 g.) (Oxo Ltd.), peptone (10 g.), NaCl (5 g.), agar (20 g.) and water (1 l.)]. The plates were examined after incubation at 25° for 48 hr.

Enzyme preparations. 'Carterzymes' ADFP 3 and C.Z. 103 were supplied by H. W. Carter and Co. Ltd., Coleford, Gloucestershire, and enzyme 19 A.P. was obtained from Rohm and Haas, Co., Philadelphia, Pa., U.S.A.

RESULTS

Anatomical observations

Observation of plum trees in various orchards showed that nodules of gum were frequently present on the bark of the trunks and branches of the trees, particularly if the tissues had been damaged mechanically or if signs of disease were present as, for example, 'die-back'. Gum nodules were less common on the surface of the fruit, but these appeared much later in the season than those on the bark. Fruit with gum nodules at the distal end more often contained internal pockets of gum than those fruit with gum nodules along the ventral suture or other parts of the epicarp.

Microscopic examination of the cut surfaces of plums (picked in July) free from signs of external gummosis showed small globules of gum exuding from the severed ends of the vascular bundles rather than from the parenchymatous tissue. Initially these globules were colourless and mobile, but on standing they rapidly darkened and became highly viscous. In an atmosphere of nitrogen the globules remained colourless, and the increase in viscosity of of the gum was inhibited by a water-saturated atmosphere.

Sections through the mesocarp which were stained with a mixture of Congo red and chrysoidin revealed the presence of gum in the cells adjacent to the vascular bundles and also in the phloem itself. Gum nodules were invariably produced when the fruit epicarp was punctured with a sterile needle. The time taken for the gum to appear after the skin was punctured varied from approximately 1 hr. to 2–3 days. The exudation occurred with fruit on the tree and with 'picked fruit'.

Gum in the branches and trunks of trees appeared to be associated with the bast and rarely penetrated into the wood.

An examination of gumming 'lesions' on plums in an orchard showed that various bacteria and fungi were associated with the tissues directly

beneath the external gum nodules, but the mesocarp tissues adjacent to internal pockets of gum were invariably sterile. It is probable that in the majority of cases the micro-organisms found beneath the nodules were saprophytic rather than parasitic.

Sugars of the fruit

Glucose appeared to be the main reducing substance present in the mesocarp tissues of both gumming and non-gumming fruit. Quantitative analysis (Table 1) showed that in the latter the glucose concentration was approximately 40% higher than in the former. Traces of D-xylose, which were tentatively identified by Hay & Pridham (1953) in the mesocarp, have now been confirmed by a comparison of the X-ray powder photograph of an authentic dibenzylidenedimethylacetal derivative of D-xylose (Breddy & Jones, 1945) with a similar derivative of the xylose isolated from the fruit.

In addition to glucose and xylose, sucrose, fructose and traces of raffinose were detected on paper chromatograms (cf. Bradfield & Flood, 1950).

Measurement of respiration showed that normal tissues had a significantly higher rate than gumming tissues (Table 2) and that the R.Q. values for the latter were higher than those for the normal tissue.

Table 1. *Reducing power and concentration of glucose in gumming and non-gumming fruit-mesocarp tissues*

Tissue		Reducing power (as % of glucose, based on dry wt. of tissue): Benzidine method	Somogyi method	Glucose (% based on dry wt. of tissue)
Non-gumming	1	68·8	57·7	—
	2	60·8	59·3	48·2
Gumming	1	34·2	30·7	27·0
	2	35·6	31·4	28·4

Table 2. *Respiration of gumming and non-gumming fruit-mesocarp tissues*

Tissue		Gas (μl./g. dry wt.): CO_2	O_2	R.Q.
Non-gumming	1	386	188	2·1
	2	355	187	1·9
	3	353	191	1·9
Average value		365	189	1·9
Gumming	1	306	79	3·9
	2	381	145	2·6
	3	280	103	2·7
Average value		322	109	3·0

R.Q. values for both types of tissue were greater than unity.

Some preliminary experiments designed to test the influence of gums on the growth of micro-organisms showed that the crude materials were bacteriostatic. A pure culture of an unidentified bacillus was isolated from the cotyledons of gumming almonds. When this organism was grown on a nutrient-agar plate which contained small nodules of plum-bark gum (surface-sterilized with ethanol) embedded into the medium, zones of growth inhibition were evident around each nodule. Bacilli growing on the boundaries of these zones were pleomorphic and many spore forms were present.

Chemical constitution of Victoria-plum gums

Paper-chromatographic examination of the hydrolysates of bark, fruit and stone gums showed that glucuronic acid, galactose, mannose, arabinose, xylose and rhamnose were present in all the gums, and in similar proportions with the exception of mannose, which appeared to be more prevelant in the bark gum.

Hydrolysis of fruit-surface gum followed by separation of the component sugars afforded crystalline specimens of D-galactose ($[\alpha]_D + 79°$; m.p. and mixed m.p., 156°); L-arabinose ($[\alpha]_D + 102°$; m.p., 149°, mixed m.p. 148°) and L-rhamnose hydrate ($[\alpha]_D + 10·5°$; m.p. and mixed m.p. 86°). D-Mannose ($[\alpha]_D + 11·9°$) and D-xylose ($[\alpha]_D + 20·7°$) were obtained as syrups and were characterized as the phenylhydrazone (m.p. and mixed m.p. 181–182°) and dibenzylidenedimethylacetal derivative (m.p. and mixed m.p. 190°) respectively.

The uronate fraction obtained from the hydrolysate was shown to contain at least three acidic oligosaccharides and two other compounds which co-chromatographed with glucurono-γ-lactone and 4-*O*-methylglucuronic acid respectively. The former was obtained as a crystalline compound (m.p. and mixed m.p. 173°). The mixed oligosaccharides on acid hydrolysis yielded galactose, mannose, glucuronic acid and a trace of arabinose. Examination of the hydrolysis products of the reduced oligosaccharides showed the presence of glucose, galactose, mannose, arabinose and 4-*O*-methylglucose.

Mild acid hydrolysis of hexuronic acid-containing polysaccharides with 100% recovery of the liberated monosaccharide components is rarely achieved, owing to the stability of the glycuronosidic bonds, and with strong mineral acids marked destruction of the monosaccharides liberated occurs. The conditions necessary for the optimum hydrolysis of Victoria-plum gum to a mixture of monosaccharides and aldobiouronic acids with N-H_2SO_4 were 4–6 hr. at 100° in a sealed tube. This was revealed by following the change in optical rotation during

Table 3. *Relative proportions of monosaccharides liberated on hydrolysis of plum gums in an autoclave*

Hydrolysis (15 lb./in.^{2}, 120°) was for 1 and 2 hr. respectively, with N-H_2SO_4. The values for 1 hr. hydrolysis are averages of two determinations; those for 2 hr. hydrolysis are averages of four determinations.

	D-Galactose		D-Mannose		L-Arabinose		D-Xylose	
Gum	1 hr.	2 hr.	1 hr.	2 hr.	1 hr.	2 hr.	1 hr.	2 hr.
Bark	1·0	1·0	0·18	0·71	0·66	1·51	0·27	0·24
Fruit surface	1·0	1·0	0·18	0·11	0·98	0·88	0·22	0·21

hydrolysis. In order to speed the routine analysis of the gum polysaccharides, attempts were made to shorten the hydrolysis time by carrying out the hydrolysis in an autoclave at 120° with N-H_2SO_4.

The change in optical rotation of solutions of various monosaccharides after varying periods of time was studied. They were extensively degraded under these conditions; after 2 hr., 33 % of L-arabinose and 22 % of D-galactose were lost (assuming that the degradation products had little or no optical activity). The technique is therefore of little use in determining absolute concentrations of monosaccharides liberated from polysaccharides, but it is useful when rapid qualitative comparative results are needed, particularly for hexuronic acid containing materials.

Aqueous solutions of plum gums readily undergo autohydrolysis because of the lability of the L-arabinose residues, which are presumably present as non-reducing end groups in the furanose form. For this reason, a comparative quantitative analysis was carried out with the crude gums to alleviate the loss of monosaccharides by hydrolysis during purification.

Analysis for uronic acid anhydride revealed that the fruit-surface gum contained less hexuronic acid (13 %) than the bark gum (18 %) when corrections were made for the ash and water content in both.

The relative proportions of D-mannose, L-arabinose and D-xylose to D-galactose were determined after acid hydrolysis of bark and fruit-surface gums under identical conditions (Table 3). After hydrolysis for 1 hr. there was little difference in the composition of the hydrolysis mixtures of the two types of gum except that a slightly higher concentration of L-arabinose had been liberated from the fruit-surface gum. However, after hydrolysis for 2 hr. it was evident that the bark gum contained a much higher concentration of L-arabinose and D-mannose than the fruit-surface gum.

When the hydrolyses (100°, sealed tube) were followed by measuring the optical rotations of the reaction mixtures, the results were similar for both types of gum. As the hydrolyses proceeded the reaction mixtures were examined on paper chromatograms at varying intervals. With both gums L-arabinose was liberated a few minutes after the reaction had started, and after 30 min. L-arabinose, L-rhamnose, D-galactose, D-xylose and three acidic oligosaccharides were detectable, together with traces of D-mannose. The last-named sugar gradually increased to a maximum after about 8 hr. After 5 hr. glucuronic acid was also detectable, its appearance on chromatograms coinciding with the gradual fall in intensity of the oligosaccharide spots. There appeared to be less hexuronic acid liberated from the fruit-surface gum than from the bark gum and the relative concentrations of the acidic oligosaccharides varied for the two gums.

Table 4. *Periodate oxidation of plum gums*

	Gum	Wt. of gum consuming 1 mole of periodate (g.)	Wt. of gum liberating 1 mole of formic acid (g.)
Purified	Bark	158	717
	Fruit surface	219	1225
Crude	Bark	179	726
	Fruit surface	233	1003

Attempts to degrade the polysaccharide of plum-bark gum to lower molecular-weight fragments with aqueous solutions of commercial enzyme preparations (i.e. enzyme 19A.P., Carterzyme ADFP 3 and Carterzyme C.Z. 103) failed.

Periodate oxidations of crude and purified specimens of both types of gum were carried out, and the uptake of periodate and liberation of formic acid were determined at various intervals of time. A comparison of the results for both purified and crude gums (Table 4) shows that purification by alcoholic precipitation had little degradative effect on the polysaccharides, differences in results probably being due to the varying ash content of the crude gums.

Electrophoresis

The potentialities of the Tiselius electrophoresis apparatus for the separation of mixtures of acidic polysaccharides were examined. Preliminary experiments showed that plum (var. Czar)-bark

gum could be separated from gum acacia with 0·2 M-borate–KCl buffer, pH 7·8. Similarly, a mixture of sugar-beet araban and plum-bark gum could be resolved with 0·07 M-phosphate buffer, pH 7·0.

Electrophoresis of plum-bark gum in several buffers (0·2 M-borate–KCl, pH 7·8; 0·07 M-phosphate, pH 7·0; 0·1 M-acetate, pH 6·0; 0·1 M-citrate, pH 3·0) suggested that it was homogeneous, as single peaks were observed in all cases. The boundaries migrated towards the positive electrode and in the borate buffer the rate of movement was calculated as $3{\cdot}08 \times 10^{-5}$ cm^2/v/sec. However, attempts to separate artificial mixtures of fruit-surface gum and bark gum in borate buffer failed.

Chromatography

Paper-chromatographic examination of cold methanolic extracts of various plant gums showed that in many cases traces of mono- and oligosaccharides were present (Table 5). At least three oligosaccharides were present in cherry-bark gum and small amounts (5–30 mg.) of each were isolated on Whatman no. 3MM paper with solvent (1). Each was completely hydrolysed to arabinose when heated with 0·01 N-H_2SO_4 for 1·5 hr. at 95°. The $R_{galactose}$ values of the three sugars in solvent (1) were 1·2, 0·65 and 0·30, and an application of the rule of Consden, Gordon & Martin (1944) suggested that oligosaccharides were a homologous series consisting of arabinobiose, arabinotriose and arabinotetraose respectively. The suspected disaccharide ($R_{galactose}$ 0·30; $[\alpha]_D^{25} + 112°$) gave a phenylosazone in small yield (m.p. 190°).

The three oligosaccharides detected in gum arabic had $R_{galactose}$ values (in solvent 1) of 0·46, 0·53 and 0·73. A small amount of the fastest-moving material, again isolated by partition on thick paper, was shown to be stable to 0·01 N-H_2SO_4 (95°; 1 hr.) but was completely hydrolysed to arabinose with N-H_2SO_4 under the same conditions. This oligosaccharide also co-chromatographed with an authentic specimen of 3-*O*-β-L-arabinopyranosyl-L-arabinose. Cold ammoniacal methanol also extracted galactose, arabinose, rhamnose and the above-named oligosaccharides from gum arabic.

Examination of a number of specimens of plum gum and fruit tissues for sugar phosphates yielded negative results. Inorganic phosphate only was detected.

The residues obtained from gum after strong acid hydrolysis yielded on oxidation with alkaline nitrobenzene a compound which co-chromatographed with vanillin and was detected on paper chromatograms with a 2:4-dinitrophenylhydrazine spray reagent. Both bark gum and fruit-surface gum reacted similarly.

Table 5. *Free sugars detected in plant gums*

Gum	Sugars detected
Cherry bark	Galactose, arabinose and three oligosaccharides
Arabic	Galactose, arabinose, rhamnose and three oligosaccharides
Almond bark	Arabinose and xylose
Plum bark (var. Cose Late Red)	
Sample I	Glucose, fructose and one oligosaccharide
Sample II	Sucrose, glucose and arabinose
Plum bark (var. Victoria)	Glucose, arabinose, xylose and two or three oligosaccharides

Phenolic and amino compounds

Paper chromatographic examination of the ether and ethyl acetate extracts of an aqueous solution of plum-bark gum showed the presence of four main components (*D*, *E*, *F* and *G*) with the properties listed in Table 6. Traces of these compounds were also detected in the residual aqueous solution. The properties of compound (*F*) were identical with those of phloroglucinol, both compounds giving similar u.v. spectra and having the same chromatographic behaviour. Phenolic glycosides appeared

Table 6. *Properties of phenolic compounds present in plum gums*

	Ether extract		Ethyl acetate extract	
Compounds	*D*	*E*	*F*	*G*
R_F [solvent (6)]	0·79	0·90	0·68	0·63
R_F [solvent (4)]	0·79	0·87	0·72	0·66
Fluorescence with u.v. light + NH_3	Pale blue	Pale blue	Blue	Pale blue
Colour with diazotized *p*-nitroaniline–Na_2CO_3 spray reagent	Brown	Brown	Red–brown	Yellow
Reaction with diazotized sulphanilic acid spray reagent	Orange	—	Orange	—
U.v.-absorption maxima (mμ)	257	—	268	—

to be absent from the gum. Fruit-surface gum was shown to have a chromatographically similar phenolic content. Plum-bark gum gave a positive Mitchell's test for tannins (Mitchell, 1923).

Analysis of several samples of plum gum showed that they all contained a small amount of nitrogen (0·3–0·6 %). No free amino acids could be detected, but after mild acid hydrolysis of the gum several compounds, which gave a blue with ninhydrin, were liberated.

Enzymes

Qualitative spot tests revealed the presence of peroxidase activity in both bark gum and fruit-surface gum. Preliminary experiments indicated that the benzidine–H_2O_2 spot test could be adapted as a quantitative method and that for the gum peroxidase there was a linear relationship between the blue produced and the weight of gum used. With this method it was shown that a higher peroxidase activity was associated with bark gum than with fruit-surface gum (Fig. 1). The peroxidase in solutions of gum (2 ml.; 1 %, w/v) was completely inactivated by heating for 1 min. in a boiling-water bath.

Phenolase activity in gums, as shown by the reaction with benzidine in the absence of H_2O_2 or by the spot test with catechol, was very small. The tissues of the fruit and bast, however, contained a relatively high concentration of phenolase.

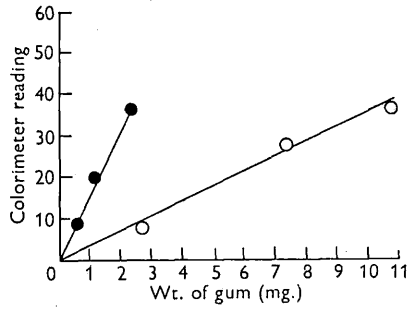


Fig. 1. Peroxidase activity in plum-bark gum (●) and fruit-surface gum (○).

Table 7. *Inorganic cations present in plum-stone gum and fruit-surface gum*

Cation	Fruit-surface gum	Stone gum
Ca	+ + +	+ + +
Mg	+ + +	+ + +
Na	+ +	+ +
K	+ +	+
Al	+ +	+ +
Sr, Ba, Be, Fe	–	–

An examination of the inorganic cations present in fruit-surface gum and stone gum of plums suggested that the two types of gum were essentially similar in mineral content. The results of this analysis are given in Table 7.

DISCUSSION

Our observations support the theory that gums are normal plant products although micro-organisms may stimulate the formation of excess of gum. Frequently excessive gum formation cannot be explained by mechanical injury or the presence of micro-organisms.

Microscopic examination of immature plum fruits showed that small amounts of gum were present in apparently sterile tissues. The hardening of the globules of gum was probably due to dehydration and not polymerization, because in a water-saturated atmosphere this increase in viscosity was prevented. In the absence of oxygen the globules remained colourless, thus suggesting that the dark colour of many gums is due to the oxidation of phenolic compounds.

The soluble sugars present in the mesocarp tissues of plum fruit included sucrose, glucose, fructose and small amounts of raffinose and xylose. All of these compounds were detected in both gumming and non-gumming fruits, but in the latter the concentration of glucose was significantly higher than in tissues showing excessive gummosis. Hexoses will be consumed in normal metabolic processes, and with gumming tissues it is probable that, in addition hexoses will also be utilized for the biosynthesis of gum polysaccharide. This may lead to their depletion, but it seems improbable that this alone would account for the fall in respiration. It seems more likely that the differences in respiration and the higher R.Q. obtained with the gumming tissues are due to the formation of a gum barrier which interferes with gaseous exchange, although partial anaerobiosis due to the thickness of the tissues would account for the high R.Q. values obtained in all cases.

It has often been stated (e.g. Hirst, 1949) that the function of the gum in the plant is to seal mechanical wounds and to 'wall-off' pockets of infection. Experiments with plum gum showed that commercial enzyme preparations, which will hydrolyse pectin and hemicelluloses, failed to degrade the gum polysaccharide. Such preparations consist of a highly reactive mixture of fungal carbohydrases. Some evidence was obtained for the presence of active bacteriostatic materials in the gum. The zones of growth inhibition which were observed when gum nodules were introduced into plate cultures of the bacillus may have been caused by the simple phenolic constituents of the gum or by

tannins. Another possibility is that the gum peroxidase has an antimicrobic action, since proteins can be oxidized and certain enzymes inactivated by this enzyme in the presence of hydrogen peroxidase (Sizer, 1953).

The monosaccharide composition of bark gums from several species of Rosaceae have been examined, e.g. cherry (Jones, 1939), damson (Hirst & Jones, 1938), egg plum (Hirst & Jones, 1947), peach (Jones, 1950) and almond (Brown, Hirst & Jones, 1948), but little has been reported on the composition of fruit-surface gums from any plant family. The Rosaceae bark gums have all been observed to contain D-glucuronic acid, D-galactose, L-arabinose and D-xylose, and some also contain D-mannose and L-rhamnose. The homogeneity of the gum polysaccharides is questionable, particularly in view of the recent work of Heidelberger, Adams & Dische (1956), who used immunological techniques to resolve gum arabic into two fractions with different monosaccharide compositions. With bark gum from egg plum, the gum nodules from different trees and varying locations have been observed to contain a constant monosaccharide composition (Hirst & Jones, 1947). Stone (1890) compared the galactose and pentose contents of the fruit-surface gum and bark gum of peach and concluded that they were similar, although the analytical methods used are open to criticism.

Analysis of plum-bark gum and fruit-surface gum revealed that the monosaccharides in both were essentially the same (4-*O*-methylglucuronic acid was not detected in bark gum, however), but the bark gum contained a greater proportion of hexuronic acid, mannose and arabinose than did the fruit-surface gum (Table 3). The differences in analytical data obtained for the 1 and 2 hr. hydrolyses can be explained by assuming that larger proportions of L-arabinose and D-mannose in the bark gum are linked to the glycosidic carbons of hexuronic acids than in the fruit-surface gum. Acid-stable aldobiouronic acids would be among the first products of hydrolysis and these units would be cleaved only by vigorous hydrolytic conditions. Support for this theory is given by the fact that acidic oligosaccharides were detected in the partial hydrolysis products of both types of gum.

The relative proportions of the monosaccharides present after hydrolysis for 1 hr. suggest that some of the D-mannose and L-arabinose units exist in a similar state of combination in both gums.

Periodate oxidation of the two gums also showed a distinct structural difference (Table 4). The results indicate that the bark-gum polysaccharide has a greater proportion of pyranosyl non-reducing end groups than the fruit-surface gum.

Qualitative analysis of the stone gum showed that it had a monosaccharide composition similar to that of the fruit-surface gum. Quantitative analysis was not possible because of the difficulty in isolating the gum free from pectin and other fruit polysaccharides. The stone gum was less soluble in water than the fruit-surface gum and it was suspected that this was due to a difference in the inorganic content of the two gums. Paper-chromatographic analysis, however, showed that the same cations were present in essentially similar proportions (Table 7). The solubility difference may be a purely physical phenomenon rather than structural differences in the macromolecules.

Colvin, Cook & Adams (1952) and Isherwood (1949) have studied the electrophoresis of polysaccharides. The former workers were able to separate an artificial mixture of sodium alginate and pectin and Isherwood was able to resolve mixtures of hemicelluloses obtained from pear cell wall. Our own preliminary experiments indicated that plant gums could be examined electrophoretically, but the length of time used for separations was limited owing to the rapid diffusion of the boundaries. With a phosphate buffer, pH 7·0, plum-bark gum was readily separated from sugar-beet araban and it could also be separated from gum arabic with borate buffer, pH 7·8. Bark gum appeared to be homogeneous in a variety of buffers of varying pH, but despite the obvious differences in the structures of bark gum and fruit-surface gum, an artificial mixture of the two could not be resolved electrophoretically. The homogeneity of plum gums is therefore still doubtful.

Small amounts of mono- and oligo-saccharides were detected in a number of different gums. Free sucrose, glucose, fructose and xylose were detected in the plant tissues; hence the gums could easily have become contaminated with these sugars. Arabinose may have arisen by autohydrolysis of arabofuranose end groups, which are common in many plant polysaccharides. In view of the mild extractive conditions, however, arabinose and the other oligosaccharides probably arose as a result of photochemical degradation of the gum polysaccharides whilst on the tree and are not substrates for gum biosynthesis.

The lability to acid of the arabinose oligosaccharides extracted from cherry gum suggested that arabofuranose units were present (Hirst, 1949). The disaccharide was probably 3-*O*- or 4-*O*-L-arabofuranosyl-L-arabinose. A $1 \rightarrow 5$ linked disaccharide is improbable owing to the high positive optical rotation. 2:5-Di-*O*-methyl-L-arabinose has been isolated from methylated cherry gum (Jones, 1947), and this could arise from $1 \rightarrow 3$ linked arabofuranose units.

The available evidence suggests that one of the oligosaccharides extracted from gum arabic is 3-*O*-β-L-arabopyranosyl-L-arabinose, a disaccharide which has been isolated from the partial hydrolysis products of several gums (Andrews, Ball & Jones, 1953; Charlson, Nunn & Stephen, 1955). The detection of this disaccharide in gum arabic is of interest in relation to the structural studies of Dillon, O'Ceallachain & O'Colla (1953), who state that one of the side chains in the gum-polysaccharide molecule must contain two arabinose units, in addition to the galactose end group, instead of a single unit as was originally suggested (Jones & Smith, 1949).

The small amount of nitrogen present in plum gums is probably all proteinaceous. Phenolase (oxidase) and peroxidase have been observed in gum arabic (Laursen, 1932) and various colorimetric tests have confirmed the presence of peroxidase in plum gums, although the phenolase activity appeared to be low.

The bark gum is invariably darker in colour than the fruit-surface gum and this may be due to the higher peroxidase activity in the former, which results in an increase in coloured phenolic oxidation products. In this connexion it is interesting that oxidized phenolic compounds can be antimicrobic (R. J. W. Byrde, A. H. Fielding & A. M. Williams, unpublished results).

Paper chromatography showed that many of the phenolic materials found in the fruit-mesocarp tissues of plum were not present in bark gum or fruit-surface gum. The fruit tissues contained a large number of compounds which, on paper chromatograms, reacted with the diazotized *p*-nitroaniline–sodium carbonate spray reagent and fluoresced under u.v. light. Evidence was also obtained for the presence of a number of phenolic glycosides. The gums, however, contained only a small number of phenolic constituents and phenolic glycosides were not apparent. It is tempting to suggest that the phenolic glycosides are utilized for the biosynthesis of gum polysaccharide, with the liberation of the phenolic aglycones. There is no direct evidence for this hypothesis, however.

With regard to the biosynthetic route of gum-polysaccharide formation the analytical results show that the plum-bark gum and fruit-surface gum have different compositions and may therefore be formed by different processes, although similar repeating units, synthesized by the same pathways, could be present in both gums.

SUMMARY

1. The composition, function and formation of gum has been studied with particular reference to the plum tree (var. Victoria).

2. The polysaccharide moiety of the gum exuded on the fruit surface was composed of D-galactose, D-mannose, L-arabinose, D-xylose, L-rhamnose, glucuronic acid and probably traces of 4-*O*-methylglucuronic acid. The bark gum was similarly constituted but contained higher proportions of D-mannose, L-arabinose and hexuronic acid. Periodate oxidation of the two types of gum suggested structural differences though their electrophoretic mobilities were identical.

3. Crude bark gum and fruit-surface gum contain the enzyme peroxidase, phenolic compounds and a 'lignin-like' substance and some inorganic material. Gums from several different species of plants were observed to contain small amounts of mono- and oligo-saccharides.

We wish to thank the Directors of the Agricultural and Horticultural Research Station, Long Ashton, Bristol, and of the Fruit and Vegetable Canning and Quick Freezing Research Association, Chipping Campden, Gloucestershire, for the facilities placed at our disposal, and H. W. Carter and Co. Ltd., Coleford, Gloucestershire, for gifts of enzyme preparations.

The inorganic analyses were carried out by Dr D. B. Powell and the organic analyses by Mr B. S. Noyes.

The work described forms part of the programme of the Department of Scientific and Industrial Research.

REFERENCES

Anderson, D. M. W., Greenwood, C. T. & Hirst, E. L. (1955). *J. chem. Soc.* p. 225.

Andrews, P., Ball, D. H. & Jones, J. K. N. (1953). *J. chem. Soc.* p. 4090.

Bradfield, A. E. & Flood, A. E. (1950). *Nature, Lond.*, **166**, 264.

Breddy, L. J. & Jones, J. K. N. (1945). *J. chem. Soc.* p. 738.

Brown, F., Hirst, E. L. & Jones, K. J. N. (1948). *J. chem. Soc.* p. 1677.

Butler, E. J. & Jones, S. G. (1949). *Plant Pathology.* London: Macmillan and Co. Ltd.

Ceruti, A. & Scurti, J. (1954). *Annu. Staz. chim.-agr. Torino*, **18**, 249.

Charlson, A. J., Nunn, J. R. & Stephen, A. M. (1955). *J. chem. Soc.* p. 1428.

Colvin, J. R., Cook, W. H. & Adams, G. A. (1952). *Canad. J. Chem.* **30**, 603.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Dillon, T., O'Ceallachain, D. F. & O'Colla, P. (1953). *Proc. R. Irish Acad.* **55** B, 331.

Dixon, M. (1951). *Manometric Methods.* Cambridge University Press.

Evans, R. A., Parr, W. H. & Evans, W. C. (1949). *Nature, Lond.*, **164**, 674.

Freudenberg, K., Lautsch, W. & Engler, K. (1940). *Ber. dtsch. chem. Ges.* **73** B, 167.

Grieg-Smith, R. (1904). *J. Soc. chem. Ind., Lond.*, **23**, 105, 972.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Hay, J. G. & Pridham, J. B. (1953). *Nature, Lond.*, **172**, 207.
Heidelberger, M., Adams, J. & Dische, Z. (1956). *J. Amer. chem. Soc.* **78**, 2853.
Higgins, B. B. (1919). *Rep. Ga agric. Exp. Sta.* **127**, 21.
Hirst, E. L. (1949). *J. chem. Soc.* p. 522.
Hirst, E. L. & Jones, J. K. N. (1938). *J. chem. Soc.* p. 1174.
Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1064.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Isherwood, F. A. (1949). *Abstr. 1st int. Congr. Biochem., Cambridge*, no. 339/11.
Johannson, A., Lindberg, B. & Theander, O. (1954). *Svensk Papp-Tidn.* **57**, 41.
Jones, J. K. N. (1939). *J. chem. Soc.* p. 558.
Jones, J. K. N. (1947). *J. chem. Soc.* p. 1055.
Jones, J. K. N. (1950). *J. chem. Soc.* p. 534.
Jones, J. K. N. & Pridham, J. B. (1954). *Biochem. J.* **58**, 288.
Jones, J. K. N. & Smith, F. (1949). *Advanc. Carbohyd. Chem.* **4**, 250.
Laursen, A. (1932). *Dansk Tidsskr. Farm.* **6**, **54**. Quoted in *Chem. Abstr.* (1932), **26**, 3873.
Lythgoe, B. & Trippett, S. (1950). *J. chem. Soc.* p. 1983.
Madeling, W. (1911). *Hoppe-Seyl. Z.* **71**, 204.
Mitchell, C. A. (1923). *Analyst*, **48**, 2.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Pollard, F. H. & McOmie, J. F. W. (1953). *Chromatographic Methods of Inorganic Analysis*. London: Butterworths.
Pridham, J. B. (1954). *Biochem. J.* **57**, xxviii.
Sands, L. & Gary, W. Y. (1933). *J. biol. Chem.* **101**, 573.
Schwartz, J. C. P. (1954). *Chem. & Ind.* p. 1000.
Sizer, I. W. (1953). *Advanc. Enzymol.* **14**, 158.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Stone, W. E. (1890). *Amer. chem. J.* **12**, 435.
Stone, J. E. & Blundell, M. J. (1951). *Analyt. Chem.* **23**, 771.
Swain, T. (1953). *Biochem. J.* **53**, 201.
Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21, 44.

A9

[*Reprinted from the Proceedings of the Biochemical Society*, 17 *June* 1966.
Biochem. J., 1966, Vol. 100, No. 2, 21P.]

A Preliminary Study on the Biosynthesis of Hemicelluloses

By J. B. PRIDHAM and W. Z. HASSID. (*Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey and Department of Biochemistry, University of California, Berkeley, California*)

Observations on the biosynthesis of higher plant polysaccharides using nucleoside diphosphate aldose derivatives as donor substrates has been largely confined to the homoglycans. Thus the major precursors of starch, callose and cellulose appear to be ADP-glucose, UDP-glucose and GDP-glucose respectively (see review by Nordin & Kirkwood, 1965). UDP-glucose is reported to be a better substrate for cellulose formation in lupin than GDP-glucose (Brummond & Gibbons, 1964, 1965). In addition, Villemez, Lin & Hassid (1965) have recently reported a synthesis of the polygalacturonic acid of pectin using UDP-galacturonic acid and a cell-free enzyme preparation from *Phaseolus vulgaris*.

Little information is available regarding the polymerization mechanisms involved in the formation of complex polysaccharides such as the plant gums and hemicelluloses. Experiments by Feingold, Neufeld & Hassid (1959) showed that a soluble enzyme preparation from asparagus catalysed single-step transfer reactions from UDP-xylose to xylose oligosaccharides.

Particulate fractions (20000*g*), from 3–4-day-old maize (*Zea mays*) seedlings incorporated up to 16% [^{14}C]pentose using UDP-[^{14}C]pentose (a 1:1 mixture of UDP-[^{14}C]xylose and UDP-[^{14}C]arabinose uniformly labelled in the pentosyl moieties) as substrate. No incorporation was observed using [^{14}C]D-xylose 1-phosphate as a substrate. A typical incubation mixture contained particulate preparation from 3–4g. of shoots in 0·7ml. of tris-HCl buffer, pH7·5, UDP-pentose (0·15μc; 107μc per μmole) and ATP (2·5μmoles). The mixture was kept at 25° for 1hr. by which time the incorporation of radioactivity into the product had ceased. Incorporation was assayed by filtering on millipore filters, washing with ethanol and counting radioactivity in the insoluble residues on the filters. The particulate fraction was highly unstable but could be kept active at −20° in 0·2M-sodium cacodylate buffer (pH7·5) for several days.

The labelled product formed was insoluble in organic solvents, very soluble in dilute alkali and slightly soluble in cold water. Complete acid hydrolysis yielded labelled arabinose and xylose in approximately equal proportions. When UDP-[^{14}C]xylose was used as a substrate the product again gave both arabinose and xylose on hydrolysis indicating the presence of an epimerase in the particulate preparation. Partial acid hydrolysis of the polymer formed from UDP-[^{14}C]pentose and chromatographic fractionation of the products yielded an immobile radioactive residue and several labelled compounds with mobilities characteristics of oligosaccharides with degrees of polymerization up to 4 or 5. Three of these were tentatively identified as 4-*O*-β-D-xylopyranosyl-D-xylose, a disaccharide of arabinose and xylose and a trisaccharide of two xylose and one arabinose residues.

It is very probable that UDP-xylose and UDP-arabinose can function as pentose donors for arabioxylan synthesis *in vivo* although the possible involvement of other nucleotide derivatives of these pentoses should be borne in mind. It is interesting to note that Feather & Whistler (1962) have isolated a corn germ hemicellulose composed almost entirely of equal amounts of L-arabinose and D-xylose.

Dr D. S. Feingold kindly provided a specimen of UDP-[^{14}C]-xylose. This research was supported in part by a research grant (A-1418) from the National Institutes of Health, U.S. Public Health Service, and a research grant from the National Science Foundation (G-23763).

Brummond, D. O. & Gibbons, A. P. (1964). *Biochem. biophys. Res. Commun.* **17**, 156.
Brummond, D. O. & Gibbons, A. P. (1965). *Biochem. Z.* **342**, 318.
Feather, M. S. & Whistler, R. L. (1962). *Arch. Biochem. Biophys.* **98**, 111.
Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1959). *J. biol. Chem.* **234**, 488.
Nordin, J. H. & Kirkwood, S. (1965). *Ann. Rev. Plant Physiol.* **16**, 393.
Villemez, C. L., Lin, T-Y., & Hassid, W. Z. (1965). *Proc. Nat. Acad. Sci., U.S.* **54**, 1626.

A10(a)

A81

Studies with glycogens from the livers of human foetuses and young children

E. J. BOURNE, ANNE MCLEAN and J. B. PRIDHAM (Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey, Great Britain)

There is some difference of opinion in the literature regarding the first appearance of glycogen in normal, human, foetal livers. Kitamura (1942), using histochemical techniques, found that the deposition began in the 17th week of the gestation period and Shapovalov (1961, 1962) states that the polysaccharide appears in liver cells as early as the 6th week. In contrast to these reports Lelong and his co-workers (1951) were unable to detect glycogen until a few weeks before term.

In this present study, with foetal livers ranging in age from $11\frac{1}{2}$ to 26 weeks, glycogen could only be extracted in detectable amounts with trichloroacetic acid after $13\frac{1}{2}$ weeks. During the 25th and 26th weeks there appeared to be a rapid increase in glycogen storage. This corresponds to the behaviour of other mammalian species where an increase in storage has been noted at a fairly late stage in the gestation period (e.g. Jacquot, 1959; Ducommun-Lehmann, 1951; Nemeth, 1954). The structures of the foetal glycogens have been examined using α-amylolysis and β-amylolysis procedures. Average chain lengths ($\overline{CL}$) vary from 12·5 to 14·5 D-glucose units and average exterior chain lengths ($\overline{ECL}$) from 7·7 to 10·2 units. Further confirmation that these glycogens possessed normal structures was obtained using concanavalin A and iodine-staining techniques. The structures of glycogen specimens from the livers of children ranging in age from 6 weeks to 11 years were also shown to be normal using the same analytical methods.

Phosphoglucomutase (D-glucose-1,6-diphosphate : D-glucose-1-phosphate phosphotransferase) and phosphoglucoisomerase (D-glucose-6-phosphate ketol isomerase) activities in foetal livers have been measured and both appear to be somewhat lower than in normal adult liver. No glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase) could be detected.

The significance of these findings will be discussed.

We are indebted to Dr Barbara E. Clayton (Hospital for Sick Children, London) for her interest and assistance in obtaining liver specimens. A.M. received financial support from the Medical Research Council.

Ducommun-Lehmann, S. (1951). *Acta anat.* **12**, 286
Jacquot, R. (1959). *J. Physiol., Paris*, **51**, 655
Kitamura, K. (1942). *Sanka Fujinka Kujo* **25**, 668; (1948) *Chem. Abstr.* **42**, 5537
Lelong, M., Rossier, A. & Laumonier, R. (1951). *Compt. rend. Soc. Biol.* **145**, 523
Nemeth, A. M. (1954). *J. Biol. Chem.* **208**, 765, 773
Shapovalov, U. N. (1961). *Arkh. Anat. Gistol. i Embriol.* **40**, 34; (1962) *ibid.* **42**, 46

A 10(b)

Biochem. J. (1966) 98, 678
Printed in Great Britain

The Structure and Deposition of Human-Liver Glycogens

BY E. J. BOURNE, ANNE McLEAN* AND J. B. PRIDHAM
Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 24 *August* 1965)

1. Glycogen was extracted from and the amount determined in human foetal livers ranging in age from 13½ to 26 weeks. 2. The detailed structures of human foetal- and child-liver glycogens were examined and shown to be essentially the same. 3. The deposition of glycogen in different mammalian species is discussed.

Most studies on glycogen deposition in the foetal liver have been made on laboratory animals, e.g. rats (Jacquot, 1959), guinea pigs (Ducommun-Lehmann, 1951; Nemeth, 1954), rabbits (Jost & Jacquot, 1955) and lambs and monkeys (Shelley, 1960). In all cases the initial concentrations of the polysaccharide are very low and they tend to rise sharply in the later stages of gestation, until at birth they have reached or surpassed the normal adult level. Such studies as have been made with human foetal material (e.g. Kitamura, 1942; Shapovalov, 1961, 1962) employed histochemical techniques and indicated that a similar pattern of development occurs. Histochemical techniques should, however, be accepted with caution, as they may detect polysaccharides other than glycogens, and there is controversy about the first appearance of glycogen in the liver tissues. No determinations of the detailed structures, e.g. measurements of CL(av.)† and ECL(av.), of normal human foetal glycogens have previously been made. Data on the structures of other mammalian foetal glycogens are also limited. Chain lengths of 11 D-glucose units for a pig-liver glycogen (Manners & Archibald, 1957), 13 units for a sheep-liver glycogen (Bell & Manners, 1952) and 11·5 units for a guinea-pig-liver glycogen (Illingworth, Cori & Larner, 1952) have been reported, but no indications of the foetal ages were given. The most comprehensive survey is that by Warren & Whittaker (1959), who studied the structure of glycogens from foetal goat livers and obtained CL(av.) and ECL(av.) values in the normal range, i.e. 10–14 and 7–9 D-glucose units respectively.

* Present address: Experimental Haematology Research Unit, Wright–Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2.

† Abbreviations: CL(av.), average chain length; ECL(av.), average exterior chain length; ICL(av.), average interior chain length, i.e. CL(av.)−ECL(av.)−1.

The present study was undertaken in an attempt to determine the earliest stages at which recognizable glycogens may be isolated from the human foetal liver, and to examine the detailed structures of these polysaccharides and compare them with glycogens from more mature liver tissues. A preliminary account of this work has been given (Bourne, McLean & Pridham, 1964).

METHODS AND MATERIALS

Human liver specimens. These specimens, both foetal and mature, were kindly provided by Dr B. Clayton, The Hospital for Sick Children, Great Ormond Street, London, W.C. 1. These were cooled to −20° as rapidly as possible after removal from the body, and stored at this temperature.

β-Amylase. This was obtained from the Wallerstein Co., Mariners Harbor, Staten Island, N.Y., U.S.A. The units of activity are expressed as mg. of maltose liberated by 1 mg. of enzyme under the conditions specified by Hobson, Whelan & Peat (1950) but at a temperature of 30°.

α-Amylase. This was prepared by the method of Fischer & Stein (1954). The crystallization stage was omitted, and the enzyme freeze-dried in 0·2M-sodium citrate buffer, pH 7·0 (Liddle & Manners, 1957). The unit of activity is the quantity of enzyme that liberates 1 mg. of apparent maltose from 1 ml. of starch solution (1%, w/v) in 3 min. at 35° (Manners & Wright, 1962).

Glycogen extraction. Homogenization with cold aq. 10% (w/v) trichloroacetic acid solution was employed as the general method (Bloom, Lewis, Schumpert & Shen, 1950). The glycogen was then precipitated from the extract with ethanol and centrifuged, and the polysaccharide was washed with ethanol followed by ether and finally dried under reduced pressure in the presence of P_2O_5.

Determination of purity of glycogens. Glycogens were hydrolysed with 2N-H_2SO_4 at 100° for 2 hr. After appropriate dilution with water, portions were assayed for glucose by the method of Somogyi (1952).

Determination of CL(av.). Samples were analysed by incubation with α-amylase as described by Manners & Wright (1962), except that the incubation time was 15 hr.; 4 units of α-amylase/mg. of glycogen were used. Apparent

maltose production was measured by the method of Park & Johnson (1949). Larger samples were assayed by periodate oxidation (Potter & Hassid, 1948) with the modification due to Manners & Archibald (1957).

Determination of ECL(av.). When relatively large amounts of glycogen were available, a 30mg. specimen was incubated with β-amylase (1800 units) and 0·2M-sodium acetate buffer, pH4·6 (5ml.), made up to 25ml. with water, at 30° for 24hr. (Bell & Manners, 1952). The maltose liberated was determined by the method of Somogyi (1952).

For the smaller samples the digests were reduced to glycogen (1mg.), β-amylase (50–70 units) and 0·2M-sodium acetate buffer, pH4·6 (0·4ml.), in a total volume of 10ml. After suitable dilution, the maltose was measured by the method of Park & Johnson (1949).

Measurement of absorption spectra of glycogen–iodine complexes. Glycogen (2·5mg.) in iodine solution (5ml. of 0·1% I_2 in 1% KI) was made up to 25ml. with water. The absorption spectrum was measured in the range 400–700mμ in a Unicam SP.500 spectrophotometer with 1cm. glass cells. The reference solution used was 0·02% I_2 in aq. 0·2% KI.

Partial acid hydrolysis. Partial hydrolysis of glycogen was carried out by using the conditions of Wolfrom, Lassettre & O'Neill (1951). The neutralized hydrolysates were examined on paper chromatograms with ethyl acetate–acetic acid–water (9:2:2, by vol.) as solvent, and the sugars located with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950). The hydrolysates, after reduction with $NaBH_4$, were also examined by paper electrophoresis with 0·062M-sodium molybdate solution as electrolyte (Bourne, Hutson & Weigel, 1961).

RESULTS AND DISCUSSION

Foetal livers ranging in age from 11½ to 26 weeks were examined. In most cases, liver tissues of the same foetal age were pooled to obtain sufficient material for analysis. Foetal livers aged 11½ and 12 weeks gave only traces of precipitate when extracted with trichloroacetic acid and then treated with ethanol. Partial acid hydrolysis of these precipitates, followed by chromatography and electrophoresis, revealed no reducing sugars, whereas the treatment of glycogen from a 26-week human foetal liver yielded glucose, maltose and isomaltose, thus revealing the presence of the expected α-(1→4)- and α-(1→6)-glucosidic linkages in the polysaccharide. The nature of the precipitates from the early foetal livers was not established.

Milligram quantities of glycogen were obtained from foetal livers at 13½ weeks (Table 1) and a sharp increase in the amount deposited was observed in 25–26-week livers.

The chemical and enzymic methods employed in this study demonstrated that the detailed structures of these glycogens and those from the livers of children aged from 6 weeks to 11 years (Table 2) were essentially the same. Most of the foetal glycogens (Table 1) were within the normal range of variation (see Manners, 1957). The values for CL(av.) varied from 12·5 to 14·5 D-glucose units (average 13·7) and those for ECL(av.) from 7·7 to 10·2 units (average 8·8). An absorption maximum of 470mμ for an iodine complex with the 26-week foetal glycogen fell within the normal range 420–490mμ (Manners, 1957), as did those for the other glycogen samples examined in this way (Table 3). This provided additional evidence that the foetal glycogen had a normal structure.

The CL(av.) values for the child-liver glycogens fell within the limits of 12·6 and 15·2 D-glucose units (average 13·3) and the ECL(av.) values ranged from 7·9 to 9·3 units (average 8·7) (Table 2). A CL(av.) value of 15·2 D-glucose units for the glycogen from 7-week liver, although high, can be accounted for by the fact that there is an intrinsic error of ±1 D-glucose unit in the method employed.

Kitamura (1942) suggested that glycogen deposition first occurs in human foetal liver at 17 weeks and that the amount begins to increase sharply

Table 1. *Structures of human foetal-liver glycogens*

Foetal age (weeks)	Fresh wt. of liver sample (mg.)	Purity of glycogen (%)	Wt. of glycogen (mg.)	Glycogen recovered from liver (%)	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)
13½	420	51·0	1·65	0·39	14·1	9·2	3·9
16	846	45·2	1·95	0·23	13·0	10·2	1·8
17	929	58·0	2·26	0·24	13·5	8·7	3·8
18½	554	58·0	2·55	0·46	12·5	7·7	3·8
20	870	22·0	2·57	0·30	14·5	—	—
21½	465	27·2	2·23	0·48	13·7	9·1	3·6
21½	1567	68·0	3·13	0·20	13·6	8·0	4·6
23½	1944	60·0	4·68	0·24	14·1	9·0	4·1
25	246	83·5	1·75	0·71	14·0	8·7	4·3
26	180	66·3	3·05	1·70	13·5	8·4	4·1
26	28·33g.	86·6	320	1·13	13·9*	8·9	4·0

* CL(av.) by periodate oxidation = 13·4 D-glucose units.

Table 2. *Structures of liver glycogens obtained from children*

Age of patient	Fresh wt. of liver sample (mg.)	Purity of glycogen (%)	Wt. of glycogen (mg.)	Glycogen recovered from liver (%)	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)
6 weeks	50	45·0	1·4	2·80	—	*	—
7 weeks	566	62·5	4·5	0·80	15·2	9·2	5·0
8 weeks	44	57·3	2·0	4·55	12·6	8·9	2·7
12 weeks	1090	85·3	19·7	1·81	13·4	7·9	4·5
12 weeks	1298	69·7	15·9	1·23	12·9	8·3	3·6
14 weeks	359	67·6	4·3	1·20	13·9	9·3	3·6
20 months	189	45·0	1·4	0·74	13·1	9·2	2·9
28 months	235	98·6	13·2	5·62	12·7	8·6	3·1
11 years	502	76·6	8·4	1·67	12·9	8·2	3·7

* This sample gave 45% conversion into maltose (the normal range is 40–50% conversion).

Table 3. *Wavelengths of absorption maxima of glycogen–iodine complexes*

Source of liver glycogen	CL(av.) by periodate oxidation	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)	$\lambda_{max.}$ (mμ)
26-week human foetus	13·4	13·9	8·9	4·0	470
Baboon	13·3	13·3	8·3	4·0	435
Mouse	13·1	13·8	8·6	3·9	455
Rabbit	13·9	12·6	8·1	4·2	470

at 26 weeks. Claims have been made that glycogen is present in the liver as early as 6 weeks (Shapovalov, 1961, 1962), but both conclusions were based on histochemical studies. Mackay, Adams, Hertig & Danziger (1956) have, however, suggested that a 4-week foetal liver is almost ready to assume the function of glycogen storage. At this stage the yolk sac still fulfils many of the liver's functions, such as glycogen storage, but has begun to transfer its enzyme systems to the liver. Thus 5-nucleotidase and non-specific esterase begin to appear in the liver and decrease in activity in the yolk sac, and Mackay *et al.* (1956) suggest on this basis that the function of glycogen storage will soon be taken over by the liver. In contrast, Lelong, Rossier & Laumonier (1951) could not detect glycogen in human foetal liver, by histochemical techniques, until a few weeks before birth.

The methods employed in the present study are, of course, too insensitive to reveal very small amounts of glycogen, and we believe that the polysaccharide, and hence the enzyme system necessary for its synthesis, is present in foetal livers younger than 13½ weeks.

The stage at which glycogen storage begins to increase rapidly appears to bear some relation to the class of mammal. In rats, guinea pigs and rabbits (rodents) it occurs very late in gestation, i.e. after 90% (Jacquot, 1959), 80% (Ducommun-Lehmann, 1951) and 75% (Jost & Jacquot, 1955) respectively of the gestation period has elapsed. In primates it occurs at a much earlier stage, for example in man after 66% of gestation has elapsed, and in Rhesus monkeys (Shelley, 1960) glycogen deposition is already high at 113 days (67% of gestation). Ungulates vary widely, the rapid increase occurring after 88% of gestation in pigs (Mendel & Leavenworth, 1907; Aron, 1922) and 61% in sheep (Shelley, 1960). Since the pituitary and adrenal hormones and insulin stimulate glycogen synthesis, it appears that the hormonal systems of primates and probably ungulates mature earlier than those of the rodents. Newborn and full-term infants respond, by pituitary discharge of adrenocorticotrophic hormone, to adrenaline (Jailer, Wong & Engle, 1951), whereas rats and mice do not respond until the eighth and eleventh days of life respectively (Jailer, 1950; Thompson & Blount, 1954), but direct comparative evidence of hormonal activities during the foetal stage is lacking.

We thank Dr B. Clayton for advice and encouragement. A. McL. was in receipt of a Medical Research Council Grant.

REFERENCES

Aron, M. (1922). *Bull. Soc. Chim. biol., Paris*, **4**, 209.
Bell, D. J. & Manners, D. J. (1952). *J. chem. Soc.* p. 3641.
Bloom, W. L., Lewis, G. T., Schumpert, M. Z. & Shen, T. (1950). *J. biol. Chem.* **188**, 631.

Bourne, E. J., Hutson, D. H. & Weigel, H. (1961). *J. chem. Soc.* p. 35.
Bourne, E. J., McLean, A. & Pridham, J. B. (1964). *Abstr. 1st Meet. Fed. Europ. biochem. Soc., Lond.*, A81, p. 65.
Ducommun-Lehmann, S. (1951). *Acta anat.* **12**, 286.
Fischer, E. H. & Stein, E. A. (1954). *Arch. Sci., Genève*, **7**, 131.
Hobson, P. N., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3566.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Illingworth, B., Cori, G. T. & Larner, J. (1952). *J. biol. Chem.* **199**, 631.
Jacquot, R. (1959). *J. Physiol. Path. gén.* **51**, 655.
Jailer, J. W. (1950). *Endocrinology*, **46**, 420.
Jailer, J. W., Wong, A. S. H. & Engle, E. T. (1951). *J. clin. Endocrin.* **11**, 186.
Jost, A. & Jacquot, R. (1955). *Ann. Endocr., Paris*, **16**, 849.
Kitamura, K. (1942). *Sanka Fujinka Kujo* (*Obstet. Gynecol. Mem., Kyoto*), **25**, 688; cited in *Chem. Abstr.* (1948) **42**, 6527c.
Lelong, M., Rossier, A. & Laumonier, R. (1951). *C.R. Soc. Biol., Paris*, **145**, 523.
Liddle, A. M. & Manners, D. J. (1957). *J. chem. Soc.* p. 3432.
Mackay, D. G., Adams, E. C., Hertig, A. & Danziger, S. (1956). *Anat. Rec.* **126**, 433.
Manners, D. J. (1957). *Advanc. Carbohyd. Chem.* **12**, 265.
Manners, D. J. & Archibald, A. R. (1957). *J. chem. Soc.* p. 2205.
Manners, D. J. & Wright, A. (1962). *J. chem. Soc.* p. 1597.
Mendel, L. B. & Leavenworth, C. S. (1907). *Amer. J. Physiol.* **20**, 117.
Nemeth, A. M. (1954). *J. biol. Chem.* **208**, 773.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Potter, A. L. & Hassid, W. Z. (1948). *J. Amer. chem. Soc.* **70**, 3488.
Shapovalov, U. N. (1961). *Arkh. Anat. Gist. Embr.* **40**, 34.
Shapovalov, U. N. (1962). *Arkh. Anat. Gist. Embr.* **42**, 46.
Shelley, H. J. (1960). *J. Physiol.* **153**, 527.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Thompson, J. C. & Blount, R. F. (1954). *Endocrinology*, **54**, 620.
Warren, F. L. & Whittaker, M. (1959). *Biochem. J.* **72**, 288.
Wolfrom, M. L., Lassettre, E. N. & O'Neill, A. N. (1951). *J. Amer. chem. Soc.* **73**, 595.

(*Reprinted from Nature, Vol.* 182, *pp.* 1687–1688, *Dec.* 13, 1958)

Occurrence and Metabolism of Oligosaccharides in the Broad Bean (*Vicia faba*)

PAPER chromatographic analysis of aqueous methanolic (75 per cent v/v) extracts of dormant broad bean seeds using (*A*) ethyl acetate/acetic acid/formic acid/water (9·0 : 1·5 : 0·5 : 2·0 v/v) and (*B*) ethyl acetate/pyridine/water (10 : 4 : 3 v/v) as solvents with *p*-anisidine hydrochloride as spray reagent[1] showed that both the embryos and cotyledons contained small amounts of raffinose, glucose and fructose together with relatively high concentrations of sucrose and higher molecular weight oligosaccharides. Traces of xylose were also detected (cf. Kawamura, Tsuboi and Nakamura[2]). The testa contained only glucose and fructose.

A large-scale extract of whole beans was concentrated to a syrup and fractionated on a carbon–'Celite' column[3]. The oligosaccharides present were identified as: sucrose [m.p. 178° C. (anhydrous), $[\alpha]_D^{20}$ + 64·3 (conc. 2·8), octaacetate m.p. and mixed m.p. 84–85° C.]; raffinose [m.p. 117–120° C. (anhydrous), $[\alpha]_D^{20}$ + 123·5 (conc. 1·1)]; stachyose [$[\alpha]_D^{20}$ + 145° (conc. 1·5, anhydrous), consumed 7·0 moles of periodate per mole] and verbascose [infrared spectrum identical with that of authentic verbascose; consumed 8·8 moles of periodate per mole]. The identity of the oligosaccharides was further proved by a comparison of their electrophoretic (0·2 *M* borate buffer, *p*H 10) and chromatographic behaviour (in solvents *A* and *B*) with that of authentic specimens, and also by a study of the hydrolysis products obtained with sulphuric acid (0·5 *N*) and yeast invertase. Electrophoretic and chromatographic evidence for the presence of a tetragalactosylsucrose in the extract was also obtained.

Germination of the beans on moist filter paper (25° C.) resulted in a rapid disappearance of the higher oligosaccharides from all parts of the seeds, leaving sucrose and an increased concentration of glucose and fructose.

The carbohydrase activity of embryos and cotyledons from both dormant and germinated (5 days) beans was examined. The tissues were macerated with cold acetate buffer (0·2 *M*; *p*H 5·6) and, after centrifugation, the solutions dialysed against buffer at 5° C. The ability of these preparations to hydrolyse sucrose, raffinose (producing free galactose) and maltose (at 35° C. under toluene using 5 per cent w/v

Biochem. J. (1960) **76**, 13
Printed in Great Britain

Oligosaccharides and Associated Glycosidases in Aspen Tissues

By J. B. PRIDHAM
Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 28 *December* 1959)

The low-molecular-weight carbohydrates present in the saps of various trees have recently been reviewed by Stewart (1957). Relatively few reports on carbohydrases and transferase activities in tree tissues are to be found in the literature, however.

This paper describes studies with quaking (*Populus tremuloides*) and bigtooth (*P. grandidentata*) aspens, where, in addition to glucose and fructose (and a trace of xylose) the main sugar present in both xylem (1-year-old-xylem elements) and 'soft xylem' (newly differentiated tissue consisting of fibres, vessels and some cambial cells) is sucrose. Photomicrographs and a full description of these tissues have been given by Kremers (1957).

Small amounts of raffinose are also present, particularly in the xylem, and *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside (1^F-β-fructosylsucrose) has been identified as a component of the 'soft xylem'. Protein preparations from the cambial region of aspen show glycosidase and transferase activities.

MATERIALS AND METHODS

Healthy trees were selected in an experimental forest at the start of the growing season; they were in full leaf. Within 10–15 min. of felling, the tissues required for sugar analyses were collected and quickly placed in absolute methanol. 'Soft-xylem' material was obtained by removing the bark from the bole and then slicing off the tissue with a knife blade held flat against the surface of the wood. Xylem was subsequently removed with a spokeshave.

All concentrations are expressed as g. of solute/100 ml. of solution unless otherwise stated.

Isolation and characterization of sugars. The collected tissues were filtered off from the methanol and re-extracted with hot methanol. The two methanolic solutions were then combined and concentrated to syrups by distillation under reduced pressure. These were examined on paper chromatograms (Whatman no. 1 paper) with ethyl acetate–acetic acid–water (A; 9:2:2, by vol.), butan-1-ol–pyridine–water (B; 6:4:3, by vol.) and propan-1-ol–ethyl acetate–water (C; 7:1:2, by vol.) solvents. *p*-Anisidine–HCl and resorcinol–HCl (Hough, Jones & Wadman, 1950) were used as spray reagents.

Small quantities of suspected sucrose and raffinose were isolated from xylem extracts (*P. grandidentata*) by partition chromatography on Whatman no. 3MM paper with solvent A. Total hydrolysis of the raffinose was effected by heating at 95° with N-H_2SO_4 for 3 hr. The acid was then neutralized with $BaCO_3$ and the hydrolysate examined on paper chromatograms (solvent A). Yeast invertase (35°; 20 hr.) was used for a partial hydrolysis and the resulting products were again examined on paper chromatograms (solvents A and B).

1^F-β-Fructosylsucrose was obtained by passing an aqueous solution of the methanol extract of 'soft xylem' (*P. tremuloides*) through a small charcoal–Celite column (Andrews, Hough & Powell, 1956). The monosaccharides were eluted from the column with water and sucrose with aqueous 2·5% (v/v) ethanol. Elution with aqueous 50%

(v/v) ethanol removed the trisaccharide and on increasing the ethanol concentration in this fraction deliquescent crystals resulted. Recrystallization from ethanol containing a trace of water produced a stable white compound, m.p. 193°. Total acid and invertase hydrolyses were carried out as described for raffinose. With the invertase the glucose and fructose produced were determined quantitatively (Pridham, 1956). A partial acid hydrolysis of the trisaccharide was effected by heating with aqueous acetic acid (20%, v/v) for 6 hr. at 45° and the hydrolysate was examined on paper chromatograms (solvent A). The trisaccharide was also examined by high-voltage paper electrophoresis (0·1 M-borate buffer, pH 10·0; Gross, 1954).

Aspen-enzyme preparation. A series of different tissues were removed from the bole of a specimen of *P. grandidentata* and, after extraction with hot methanol, were analysed for N (micro-Kjeldahl method).

'Soft phloem' (180 g.; tissue consisting of young phloem and cambial elements) was removed with a knife from the inner bark of a young aspen (*P. grandidentata*) and immediately macerated with 0·1 M-phosphate buffer (pH 7·0; 800 ml.; 5°) containing sodium dithionite (0·1%), to prevent browning. The extract was filtered through glass wool, centrifuged at 3000 rev./min. and then dialysed against distilled water (5°) for 3–4 days in the presence of toluene. The resulting precipitate was removed by centrifuging. Ammonium sulphate was then added successively to the dialysed extract to 25 and 50% saturation. The precipitate resulting at the lower salt concentration was discarded but the bulk of the protein, which came down at the higher saturation, was filtered off, taken up in water and then dialysed under toluene against distilled water for 3–4 days (5°). The required amount of Na_2HPO_4 and NaH_2PO_4 to buffer the solution at pH 7·0 (0·1 M) was then added with continual stirring, the solution being kept at 5°.

The aspen-enzyme preparation (5 ml.) was incubated in the presence of toluene (37°) with phenyl-α-D-glucoside, maltose, phenyl-β-D-glucoside, cellobiose, arbutin, salicin, sucrose, raffinose, 4-*O*-β-D-xylopyranosyl-D-xylopyranose, potato starch and citrus pectin, each at a concentration of 5%. Incubations with 10% sucrose and 0·5% arbutin were also carried out, and all substrates were incubated with boiled-enzyme preparations to serve as controls. After incubation for 48 hr. the solutions were examined on paper chromatograms for sugars, with the *p*-anisidine–HCl spray reagent (solvent A). Where arbutin was used as substrate the chromatograms [butan-1-ol–ethanol–water (D; 40:11:19, by vol.) and B solvents] were also examined for phenolic compounds by the use of diazotized *p*-nitroaniline–Na_2CO_3 spray reagent (Swain, 1953).

An unidentified phenolic compound (I) which was formed with arbutin (5%) as substrate was isolated as a brown syrup by partition on Whatman no. 3 MM paper (solvent D). Compound (I) was hydrolysed with N-H_2SO_4 (2 hr.; 95°) and the hydrolysate examined on paper chromatograms with solvents A and D.

The rates of formation of compound (I) and glucose were followed by incubating aspen protein (30 mg., freeze-dried from aqueous solution) with arbutin (1·0%) in 0·1 M-phosphate buffer (10 ml.; pH 6·1) at 37°. Samples (1 ml.) were withdrawn at various intervals of time, deproteinized by the method of Sevag, Lackman & Smollens (1938) and the free glucose and (I) were determined by quantitative paper-chromatographic methods (Pridham, 1956, 1957; Fig. 1).

The effect of D-glucose (1·0%) and D-glucono-1:4-lactone (0·19%) on the formation of compound (I) was studied by incubating these compounds with arbutin (5·0%) and the aspen enzyme. Attempts were also made to synthesize *p*-hydroxyphenyl glucosides by incubating the enzyme with a mixture of quinol (1·0%) and (i) D-glucose (3·0%) and (ii) gentiobiose (3·0%).

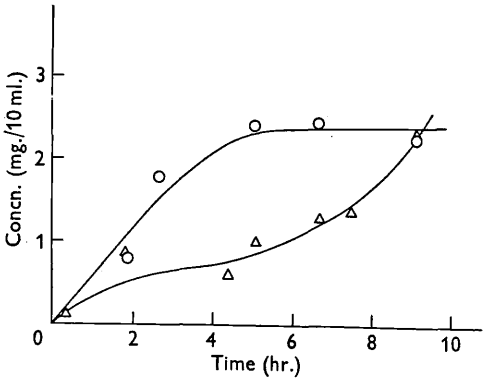


Fig. 1. Incubation of aspen enzyme with arbutin (1·0%) at pH 6·1. ○, *p*-Hydroxyphenyl-β-gentiobioside; △, free glucose.

RESULTS

Paper-chromatographic studies with methanol extracts of the xylem and 'soft xylem' of *P. tremuloides* and *P. grandidentata* showed clearly that glucose, fructose and sucrose (and generally a trace of xylose) were present in all cases. Sucrose was characterized by crystallization from aqueous ethanol [m.p. 179°; $[\alpha]_D^{25} + 64·7°$ in water (*c*, 10·0)] and by the preparation of the octa-acetate (m.p. 85–88°, mixed m.p. 86–88°).

A compound which cochromatographed with raffinose in two solvent systems was also detected; it was chiefly present in the xylem extracts. This sugar gave a yellow spot with *p*-anisidine–HCl and a pink colour with resorcinol–HCl, both of these reactions being indicative of a ketose-containing compound. Total acid hydrolysis yielded galactose, glucose and fructose and yeast invertase produced fructose and a disaccharide which cochromatographed with melibiose in acidic and basic solvent systems. Traces of high-molecular-weight sugars which may have belonged to the 'raffinose family' were also present.

In 'soft-xylem' extracts from both aspen species a sugar was detected which was chromatographically and electrophoretically indistinguishable from 1^F-β-fructosylsucrose. With these last-named techniques it was possible to distinguish between 1^F-β-fructosylsucrose and the isomeric trisaccharides 6^F-β-fructosylsucrose [*O*-α-D-glucopyranosyl-(1 → 2)-

O-β-D-fructofuranosyl-(6 → 2)-β-D-fructofuranoside] and 6^G-β-fructosylsucrose [*O*-β-D-fructofuranosyl-(2 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside]. Complete acid hydrolysis yielded only glucose and fructose and treatment with dilute aqueous acetic acid produced mainly sucrose, together with some glucose and fructose. Yeast invertase completely degraded the sugar to glucose and fructose in a molar ratio of 1:2.

A determination of the alcohol-insoluble nitrogenous compounds in various tissues from aspen (Table 1) suggested that the highest protein concentration in the bole of the tree occurred in the cambial region (cf. Stewart, 1957).

On the basis of these results a protein solution was prepared from the 'soft-phloem' tissues and tested for carbohydrase and transferase activities. The results of this study are shown in Table 2. α-Glucosidase, β-glucosidase, α-galactosidase and sucrase all appear to be present in the preparation. With sucrose at 5% concentration only glucose and fructose were formed. However, with 10% of substrate there was definite evidence for the formation of 1^F-β-fructosylsucrose after incubation for 7 hr., and after 48 hr. there was a marked increase in the amount produced. Transferase activity was also very apparent when arbutin was used as a substrate. In addition to the hydrolytic products, glucose and quinol, a slow-moving phenolic compound (I) was also detected on chromatograms [R_F values: solvent D, 0·25; solvent B, 0·38] at substrate concentrations of 0·5 and 5·0%. Compound (I) gave the same purple colour as arbutin with the diazonium reagent and was chromatographically identical to (*p*-hydroxyphenyl)-β-gentiobioside. Acid hydrolysis of compound (I) gave rise to quinol and glucose. The formation of (I) and the liberation of glucose was followed during the incubation of arbutin with aspen protein. As the concentration of compound (I) reached a maximum there was a rapid increase in the amount of free glucose in solution (Fig. 1). The inclusion of D-glucono-1:4-lactone or D-glucose in the reaction mixtures completely inhibited the formation of (I) and neither arbutin nor (I) was formed when D-glucose or gentiobiose and quinol were incubated with the aspen protein.

Table 1. *Protein nitrogen in aspen tissues*

	N (%)
P. grandidentata	
Periderm	0·56
Secondary phloem	0·21
'Soft phloem'	1·34
*P. tremuloides**	
'Soft xylem'	1·40
1-year-old xylem	0·28
2-year-old xylem	0·08

* Results obtained by Sultze (1956).

DISCUSSION

D-Glucose, D-fructose and sucrose commonly occur as free sugars in all plants. Raffinose, which has been detected chromatographically in sieve-tube exudates from a number of trees (Zimmermann, 1957) frequently accompanies sucrose in plant tissues (e.g. French, 1954; Bradfield & Flood, 1950; Cerbulis, 1955; Pridham, 1958) and D-xylose has also been detected in the free state in plants: for example, in fruits (Hay & Pridham, 1953; Ash & Reynolds, 1955) and in heartwood (Kritchevsky & Anderson, 1955).

The literature on the isomeric trisaccharides, 1^F-β-fructosylsucrose, 6^F-β-fructosylsucrose and 6^G-β-fructosylsucrose, is extensive. All these

Table 2. *Carbohydrase and transferase activities of aspen-protein solution*

Substrate	Products detected by *p*-anisidine–HCl spray reagent	
Phenyl-α-D-glucoside	Glucose	—
Maltose	Glucose and trisaccharide ($R_{glucose}$ 0·11; solvent A)	—
Phenyl-β-D-glucoside	Glucose	—
Cellobiose	Glucose and trisaccharide ($R_{glucose}$ 0·11; solvent A)	—
Sucrose	Glucose, fructose and 1^F-β-fructosylsucrose	1^F-β-Fructosylsucrose formed only with substrate concentration of 10%
Potato starch	Glucose, disaccharide and trisaccharide	Hydrolysis products present only in traces
Citrus pectin	Galacturonic acid, galactose, arabinose	Present only in small amounts
Salicin	Glucose	—
Raffinose	Sucrose, galactose, glucose and fructose	Main products over 24 hr. incubation were galactose and sucrose
4-*O*-β-D-Xylopyranosyl-D-xylopyranose	Xylose	Only slight hydrolysis

compounds have been synthesized by the action of transfructosidases from various organisms on sucrose. 1^F-β-Fructosylsucrose and 6^G-β-fructosylsucrose were prepared by Allen & Bacon (1956) with enzymes from sugar-beet leaves and other plants and by Bacon (1954) and Gross, Blanchard & Bell (1954) with yeast invertase. 1^F-β-Fructosylsucrose has also been prepared by incubating sucrose with various fungal enzymes (Pazur, 1952; Barker & Carrington, 1953; Barker, Bourne & Carrington, 1954; Bacon & Bell, 1953) and with preparations from Jerusalem-artichoke tubers (Dedonder, 1952*a*) and *Aerobacter levanicum* (Feingold, Avigad & Hestrin, 1956). With *A. levanicum* 6^F-β-fructosylsucrose was also produced, as was the case with yeast invertase and sucrose (Albon, Bell, Blanchard, Gross & Rundell, 1953; Bacon, 1954).

With regard to the natural occurrence of these compounds in plant tissues, 1^F-β-fructosylsucrose and higher homologues have been found in artichoke tubers (Bacon & Edelman, 1951; Dedonder, 1952*b*), and this trisaccharide, together with 6^G-β-fructosylsucrose, has recently been reported to be present in onion, leek, Italian ryegrass and tall oatgrass. 6^F-β-Fructosylsucrose may also occur in these grasses (Bacon, 1959) and its presence in perennial ryegrass has definitely been established by Schlubach, Lübbers & Borowski (1955). 6^G-β-Fructosylsucrose has tentatively been identified in extracts from ripe bananas by Henderson, Morton & Rawlinson (1959), and these workers have shown that banana preparations exhibiting invertase activity can synthesize fructosylsucrose derivatives from sucrose. Fructose-containing oligosaccharides have also been tentatively identified by paper-chromatographic methods in a number of other plant species. These have been listed by Bacon (1959).

In general it would appear that the fructosylsucrose derivatives are peculiar to plant tissues which have relatively high sucrose concentrations. This is particularly well illustrated with the onion, where the only scales having the oligosaccharides are those towards the centre of the bulb, which have high sucrose concentrations (Bacon, 1959). Also in the banana the formation of 6^G-β-fructosylsucrose occurs as the fruit ripens and the glucose, fructose and sucrose content increases. In aspen the 1^F-β-fructosylsucrose would appear to accumulate in the cambial region, which again has a high sucrose concentration (Kremers, 1957). Under such conditions the formation of 1^F-β-fructosylsucrose by a transfructosidase would be favoured. No trisaccharide was detected in the xylem, where the sucrose concentration is much lower; however, this could also be due to the absence of the necessary transferase in this tissue. High sucrose concentrations (10–20%) have also commonly been used to prepare fructosylsucrose derivatives (*in vitro*) with the various transfructosylases that have been mentioned, and with the aspen enzyme a substrate concentration of 10% was apparently necessary in order to produce detectable amounts of 1^F-β-fructosylsucrose within a reasonable period of incubation. No detailed search for other fructosylsucrose derivatives was undertaken and the possibility that such compounds were also present in 'soft xylem' or the aspen-protein incubation mixtures or in both should not be ignored.

A further general point is that, in the majority of cases, naturally occurring fructosylsucrose derivatives have been isolated only from plant-storage tissues. With aspen, however, the trisaccharide is present in a meristematic tissue with a high metabolic activity.

The examination of the enzymic activity of the 'soft-phloem' proteins showed that many of the commonly occurring plant glycosidases (Sumner & Myrbäck, 1950) are present in these aspen tissues. The results are supported by the observations of Sosa (1940) and Bois & Chubb (1942), who have detected invertase, maltase, emulsin and amylase in species of birch, and by Kuprevich (1949) and Meeuse (1949, 1952), who report amylase activities in other deciduous trees. Zimmermann (1958) believes that there is an α-galactosidase in sieve-tube cytoplasm. The importance and exact functions of these enzymes in trees, as in other plants, remain obscure.

Transglucosylation and transfructosylation reactions were also evident where sucrose and arbutin were used as substrates. In the latter case the mechanism would appear to be a β-glucosidase-catalysed transfer of glucose from arbutin to a second arbutin-acceptor molecule, similar reactions having been demonstrated with enzyme preparations from broad-bean and almond seeds (J. D. Anderson, L. Hough & J. B. Pridham, unpublished results). Evidence to support this mechanism is given by the fact that β-glucosides were readily hydrolysed by the protein preparation and this activity and the synthesis of (*p*-hydroxyphenyl)-β-gentiobioside were completely suppressed by D-glucono-1:4-lactone (Conchie & Levvy, 1955) and D-glucose (Jermyn, 1955), both of which can inhibit β-glucosidase. Attempts to use quinol as a direct acceptor of glucose failed; the purification procedure used for the enzyme preparation makes it unlikely that uridine diphosphate glucose is participating in the reaction.

SUMMARY

1. Sucrose is the main oligosaccharide present in both the cambial region and young-xylem elements of aspen.

2. Raffinose and 1^F-β-fructosylsucrose are also present, the former being mainly associated with the xylem and the latter with the 'soft xylem'.

3. A protein preparation from the 'soft phloem', when incubated with sucrose, produced glucose, fructose and 1^F-β-fructosylsucrose. The preparation also exhibited marked α- and β-glucosidase and α-galactosidase activities.

I thank Dr R. E. Kremers for many helpful discussions and encouragement; Dr D. Gross for useful advice; and the Institute of Paper Chemistry, Appleton, Wisconsin, U.S.A., for the facilities provided for this work. The Rhinelander Paper Company, Wisconsin, U.S.A., kindly allowed plant materials to be collected from their experimental forest.

REFERENCES

Albon, N., Bell, D. J., Blanchard, P. H., Gross, D. & Rundell, J. T. (1953). *J. chem. Soc.* p. 24.
Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200.
Andrews, P., Hough, L. & Powell, D. B. (1956). *Chem. & Ind.* p. 658.
Ash, A. S. F. & Reynolds, T. M. (1955). *Aust. J. Chem.* **8**, 276.
Bacon, J. S. D. (1954). *Biochem. J.* **57**, 320.
Bacon, J. S. D. (1959). *Biochem. J.* **73**, 507.
Bacon, J. S. D. & Bell, D. J. (1953). *J. chem. Soc.* p. 2528.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Barker, S. A., Bourne, E. J. & Carrington, T. R. (1954) *J. chem. Soc.* p. 2125.
Barker, S. A. & Carrington, T. R. (1953). *J. chem. Soc.* p. 3588.
Bois, E. & Chubb, W. O. (1942). *Canad. J. Res.* **20** B, 114.
Bradfield, A. E. & Flood, A. E. (1950). *Nature, Lond.*, **166**, 264.
Cerbulis, J. (1955). *Arch. Biochem. Biophys.* **58**, 406.
Conchie, J. & Levvy, G. A. (1955). *Biochem. J.* **61**, xvi.
Dedonder, R. (1952*a*). *Bull. Soc. Chim. biol., Paris*, **34**, 171.
Dedonder, R. (1952*b*). *Bull. Soc. Chim. biol., Paris*, **34**, 144, 157.
Feingold, D. S., Avigad, G. & Hestrin, S. (1956). *Biochem. J.* **64**, 351.
French, D. (1954). *Advanc. Carbohyd. Chem.* **9**, 149.
Gross, D. (1954). *Nature, Lond.*, **173**, 487.
Gross, D., Blanchard, P. H. & Bell, D. J. (1954). *J. chem. Soc.* p. 1727.
Hay, J. G. & Pridham, J. B. (1953). *Nature, Lond.*, **172**, 207.
Henderson, R. W., Morton, R. K. & Rawlinson, W. A. (1959). *Biochem. J.* **72**, 340.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Jermyn, M. A. (1955). *Aust. J. biol. Sci.* **8**, 577.
Kremers, R. E. (1957). *T.A.P.P.I.*, **40**, 262.
Kritchevsky, G. & Anderson, A. B. (1955). *J. Amer. chem. Soc.* **77**, 3391.
Kuprevich, V. F. (1949). *Bot. Zh.* **34**, 613.
Meeuse, D. (1949). *New Phytol.* **48**, 125.
Meeuse, D. (1952). *Acta bot. neerl.* **1**, 216.
Pazur, J. H. (1952). *J. biol. Chem.* **199**, 217.
Pridham, J. B. (1956). *Analyt. Chem.* **28**, 1967.
Pridham, J. B. (1957). *Analyt. Chem.* **29**, 1167.
Pridham, J. B. (1958). *Nature, Lond.*, **182**, 1687.
Schlubach, H. H., Lübbers, H. & Borowski, H. (1955). *Liebigs Ann.* **595**, 229.
Sevag, M. G., Lackman, D. B. & Smollens, J. (1938). *J. biol. Chem.* **124**, 425.
Sosa, A. (1940). *Ann. Chim.* **14**, 5.
Stewart. C. M. (1957). *T.A.P.P.I.*, **40**, 244.
Sultze, R. (1956). Ph.D. Thesis: Institute of Paper Chemistry, Appleton, Wisconsin, U.S.A.
Sumner, J. B. & Myrbäck, K. (1950). *The Enzymes*. New York: Academic Press Inc.
Swain, T. (1953). *Biochem. J.* **53**, 201.
Zimmermann, M. H. (1957). *Plant Physiol.* **32**, 288.
Zimmermann, M. H. (1958). In *The Physiology of Forest Trees*, p. 381. Ed. by Thimann, K. V. New York: The Ronald Press Co.

Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

THE SEPARATION AND IDENTIFICATION OF OLIGOSACCHARIDES

R. W. BAILEY* AND J. B. PRIDHAM

Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey (Great Britain)

CONTENTS

I. INTRODUCTION

The separation and identification of various types of monosaccharides by chromatographic procedures has been extensively developed since PARTRIDGE[120] first applied paper partition chromatography to the resolution of mixtures of these compounds. Identification of monosaccharides by modern paper chromatographic methods is nowadays normally reliable. The determination of enantiomers, however, is still beyond the bounds of the technique. Paper chromatography is also used extensively in examining oligosaccharide mixtures, the identification of which is of considerable importance in elucidating polysaccharide structures and in investigating many enzymic processes. In the case of oligosaccharides, however, a vast number are theoretically possible and chromatographic methods must, therefore, be used with greater caution. The aim of the present article is to describe the use of chromatography in oligosaccharide investigations and to discuss its limitations.

The review discusses firstly *paper partition chromatography* which generally provides a preliminary identification of the sugars in a mixture; secondly, the related technique of *paper electrophoresis*, and thirdly, *column chromatography* which may be used for the isolation of sufficient material for confirmatory chemical identification.

* Present address: Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand.

References p. 133.

2. PAPER CHROMATOGRAPHY

(a) *General considerations*

The paper chromatography of oligosaccharides is generally performed under similar conditions to those used for separating monosaccharides. A descending solvent technique is normally used with, in the great majority of cases, Whatman No. 1 (or an equivalent) filter paper. The simple apparatus required, consisting of glass troughs in a closed, gas-tight, glass tank, is adequately described by LEDERER AND LEDERER[107].

Compounds under examination are normally applied to the papers as aqueous or alcoholic solutions. Glass capillary tubes are commonly used for this purpose. For the application of large volumes, a camel-hair brush is recommended, particularly in the case of preparative paper chromatography where the sugars are streaked along the origin.

Strong acids and bases and high concentrations of salts should always be removed from solutions of sugars prior to chromatographic analysis, as these inorganic materials will interfere with the resolution. Desalting may be achieved by a number of techniques, the simplest being careful removal of water by distillation under reduced pressure followed by extraction of the sugars with anhydrous methanol or pyridine[116]. It should be remembered, however, that hot pyridine can epimerise reducing sugars.

Ion-exchange resins are also commonly used for desalting sugar solutions. This method is relatively safe provided the necessary precautions are taken. The solution should be treated first with the cation-exchange resin (*e.g.* Amberlite IR-120 or Dowex-2) and then with the anion-exchanger (*e.g.* Amberlite IR-4B or Dowex-50), or a mixed bed resin can be used. If this sequence is followed, high pH values, which may epimerise or in other ways affect sugars, are avoided. Sugar acids and amino-sugars are of course adsorbed by anion- and cation-exchangers respectively. Strong base resins such as Amberlite IRA-400 (OH^- form) or Dowex-2 (OH^- form) can degrade and adsorb neutral sugars and should always be used in the carbonate form[113]. Other techniques for desalting sugar solutions include electrolytic methods[49] and the use of carbon[90]. The removal of borate ions from column washings (see pp. 131, 133) or borohydride reduction reaction mixtures can be achieved by treatment with methanol[28] or methanolic HCl[42] after removal of cations with an ion-exchange resin[28], or by treatment with carbon[90]. It should be noted, however, that many sugars form borate complexes, particularly under alkaline conditions, and these may be adsorbed on to an ion-exchange resin which is used to remove borate ions.

(b) *Solvents*

Almost all solvent systems employed for the partition chromatography of sugars have water as the stationary phase and an organic solvent as the mobile phase. The one or two exceptions which employ either two organic solvents or a salt solution instead of water do not appear to be generally applicable to the chromatography of oligosaccharides. Careful equilibration of papers with the vapour phase of the solvent does not always appear to be as vital as some early authors claimed. The method of BAYLY AND BOURNE[31], in which a sheet of paper wetted with the developing solvent is

hung alongside the chromatograms being developed, appears to give suitable equilibration and was employed in the present work for evaluation of the various solvents. Precise temperature control is generally unnecessary; the main precaution in this connection is to avoid low temperatures which cause some solvent systems to separate into two phases. From the point of view of the solvent, the essential criteria are that the tank should be gas tight and of a construction which enables it to be easily cleaned.

Many of the solvents described have been developed for the resolution of monosaccharide mixtures and are not necessarily suitable for oligosaccharide separations. In resolving mixtures of these higher sugars the value of a system depends on its ability to perform one or other, or both, of the following functions:

(a) Resolution of mixtures of oligosaccharides of the same D.P.; particularly those with D.P. values of 2 or 3;

(b) Resolution of mixtures of oligosaccharides of different D.P. values; particularly homologous series.

Of the many solvent systems which have been reported to be suitable for paper chromatography of sugars, some 60 appeared to be of value for oligosaccharide separations. These have been tested by the authors, and classified (Table I) for this review, using the standard apparatus referred to above, and the following range of sugars: gentiobiose, isomaltose, maltose, cellobiose, laminaribiose, sophorose, α,α-trehalose, melibiose, lactose, isomaltotriose, maltotriose, panose, isomalto-tetraose, -pentaose, -hexaose, -heptaose, and -octaose, sucrose, raffinose, melezitose, stachyose, D-glucuronic acid, D-galacturonic acid and the di- and tri-galacturonic acids from pectin[99]. Rather than report numerous R_F and R_G values, the 46 solvents which proved useful have been listed in a more general manner, in Table I according to their ability to perform the tasks mentioned above. To give an indication of the time required for development with each solvent its speed has been classified (see footnote to Table I) according to the time in which D-glucose moves to the bottom quarter of a standard (22 cm × 44.5 cm) sheet of Whatman No. 1 paper. Likewise, in recording the ability of each solvent to resolve oligosaccharides in the isomaltose series of increasing D.P., the sugars which just move clear from the starting line when D-glucose has reached the bottom quarter of the paper have been noted. All of the solvents listed in Table I are either single-phase systems or the top layers of two-phase systems: the one exception is phenol–water solvent which is the bottom layer of a two-phase system. Only the acidic solvents were suitable for resolving the hexuronic acids and the acidic oligosaccharides.

Inspection of Table I shows that while many solvents are useful for separating sugars of different D.P. values, only a few give really good separations of closely related sugars of the same D.P. Nevertheless, sufficient solvents of acidic, neutral and basic composition are available for most purposes. The following solvent systems (where all ratios are by volume) were also tested and found to be unsuitable for the chromatography of oligosaccharides:

n-Butanol–formic acid–water (2:1:2), ethyl acetate–acetic acid–water (3:1:3), isopropanol–acetic acid–water (1:5.9:3.2), *n*-butanol–ethanol–water (4:1:5), ethyl

References p. 133.

TABLE I

SOLVENT SYSTEMS SUITABLE FOR RESOLVING OLIGOSACCHARIDE MIXTURES

No.	Solvent composition (all v/v)	Speed*	Resolution of di- and tri-saccharide mixtures**	Separation of oligo-saccharides of increasing D.P.***	Separation of oligouronides	Reference†
A. Acidic						
n-Butanol–acetic acid–water						
1.	2:1:1	F	moderate	6	good	80
2.	5.2:1.3:3.5	F	poor	6	good	129
3.	4:1:2	MS	poor	4	good	
4.	5:1:2	MS	moderate	5	good	85
5.	5:1:4	S	moderate	4	moderate	76
6.	4:1:5	S	moderate	4	moderate	16
7.	6.3:1:2	VS	moderate	3	moderate	
Ethyl acetate–acetic acid–water						
8.	2:1:2	VF	moderate	8	moderate	52
9.	10:5:6	VF	moderate	6	good	
10.	3:3:1§	VF	poor	4	poor	96
11.	9:2:2	VS	moderate	3	poor	6
Ethyl acetate–acetic acid–formic acid–water						
12.	9:1.5:0.5:2	MF	very good	4	moderate	80
n-Propanol–acetic acid–water						
13.	4:1:2	MF	poor	6	good	
Isopropanol–acetic acid–water						
14.	7:1:2	MF	moderate	5	poor	57
Benzyl alcohol–n-propanol–formic acid–water						
15.	7.2:5:2:2	MS	good	4	good	68
Phenol–water						
16.	Bottom layer	MS	moderate	3	—	120
B. Neutral solvents						
n-Butanol–ethanol–water						
17.	4:1.1:1.9	MS	good	4		5
18.	5:2:2	MS	good	6		11
19.	5.7:1.3:3.2	MS	moderate	5		76
20.	5:1:4	MS	moderate	4		55
21.	10:1:2	VS	moderate	4		57
Ethyl acetate–n-propanol–water						
22.	1:6:3	MF	poor	8		43
23.	5.7:4.2:2	MF	poor	4		2
24.	1:7:2	MF	moderate	5		57
25.	5.7:3.2:1.3	MS	very good	4		2
n-Propanol–water						
26.	4:1§	MF	poor	4		88
27.	7.8:2.2§	F	moderate	4		114

(continued on p. 118)

References p. 133.

TABLE 1 (*continued*)

No.	Solvent composition (all v/v)	Speed*	Resolution of di- and tri-saccharide mixtures**	Separation of oligo-saccharides of increasing D.P.***	Separation of oligouronides	Reference†
Isopropanol–water						
28.	9:2	MS	moderate	3		82
C. Basic solvents						
n-Butanol–ethanol–water–ammonia (used for separation of reducing sugars as N-benzylglycosylamine derivatives)						
29.	4:1:4.9:0.1	VF	good	4		31
30.	4.5:0.5:4.9:0.1	VF	very good	4		31
31.	4:1.2:2:0.1	VF	moderate	7		31
n-Butanol–pyridine–water						
32.	3:2:1.3	MF	moderate	6		141
33.	6:4:3	MS	moderate	6		93
34.	3:1.3:1.5	MS	poor	6		93
35.	10:3:3	S	moderate	5		85
36.	5:3:2	S	very good	5		11
n-Butanol–pyridine–benzene–water						
37.	5:3:1:3	MS	moderate	5		3
Ethyl acetate–pyridine–water						
38.	2:1:2	VF	moderate	6		96
39.	10:5:6	VF	moderate	6		
40.	10:4:3	F	good	5		8
41.	8:2:1	MS	moderate	4		85
42.	5:2:5	S	poor	3		93
Ethyl acetate–pyridine–water–acetone						
43.	defined by S. G.	VF	moderate	9		115
Ethyl acetate–pyridine–acetic acid–water						
44.	5:5:1:3	VF	moderate	4		77
Collidine–ethanol–water						
45.	4:3:2	VF	poor	5		93
Collidine–pyridine–water						
46.	2:1:1§	MF	poor	5		93

* VF: very fast; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in 18 h; F: fast; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in 24–30 h. MF: moderately fast; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in 36–40 h. MS: moderately slow; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in 48–60 h. S: slow; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in 72–80 h. VS: very slow; glucose moved to bottom quarter of a standard 22 × 44.5 cm chromatogram in over 96 h.

** Poor: all di- or tri-saccharides tend to move at the same rate. Moderate: only di- or tri-saccharides of markedly different structures separate. Good: sugars of closely similar structures partially separate. Very good: sugars of closely similar structures (*e.g.* maltose and cellobiose) clearly separate.

*** Figures refer to D.P. values for the oligosaccharides, in the isomaltose series, which just move clear from the starting line when D-glucose has reached the bottom quarter of a 22 × 44.5 cm standard chromatogram.

† References to some solvent systems could not be traced in the literature.

§ Sugar spots rather diffuse or streaking.

References p. 133.

acetate–ethanol–water (2:1:2), ethanol–methanol–water (9:9:2), *n*-butanol–pyridine–water (10:1:2, 9:5:8, 1:1:1 and 1:1:1.5), *n*-butanol–acetone–ammonia–water (4:5:0.5:1.5) and *n*-propanol–ammonia–water (16:3:1). These solvents were either too slow for sugars of D.P. 3, and at the same time gave very poor resolution of di- and tri-saccharide mixtures, or they caused the sugar spots to streak.

The movement in the various solvents of oligosaccharides containing amino sugar or sugar phosphate residues was not investigated for this review, as a suitable range of test sugars was not available. BARKER *et al.*[29], however, have used solvent No. 29 (omitting the benzylamine) for the resolution of the glucosamine oligosaccharides obtained by the partial hydrolysis of chitin. Solvents used in the paper chromatography of amino sugar containing disaccharides obtained from various mucopolysaccharides include *n*-butanol–pyridine–water (3:1:1[50] and No. 33[93]) and *n*-butanol–acetic acid–water (6.3:1:2.7[124] and 4.4:1.6:4[117]). Other nitrogen-containing oligosaccharides from human milk and mammary gland have been investigated with solvent Nos. 6[78], 38 and 43[115].

Little work has been carried out on the paper chromatography of oligosaccharide phosphates. However, solvents described by HARRAP[73], RUNECKLES AND KROTKOV[133], HESTRIN AND AVIGAD[77] and HORECKER, SMYRNIOTIS, AND SEEGMILLER[82] for separatting monosaccharide phosphates may be of value for sugar phosphates of higher D.P.

THOMA AND FRENCH[138] have investigated the effect of changing the proportions of a solvent mixture on its ability to separate oligosaccharides. These authors describe a method for deducing the most suitable composition of a particular solvent system for separating the members of an homologous series of oligosaccharides. The method is demonstrated with *n*-butanol–pyridine–water and ethyl acetate–acetic acid–water solvent mixtures.

Development with most systems is generally continuous for from 1–4 days. With the longer development times the solvent is allowed to drip off the serrated bottom edge of the paper. Multiple development in which the paper chromatogram is repeatedly developed for a short period, dried and then re-developed has been shown by JEANES, WISE AND DIMLER[93] to give improved separation of many mono- and some di-saccharides with some solvent systems. This technique may be well worth considering when attempting to separate closely moving oligosaccharides.

A study of the movement of an unknown oligosaccharide in a range of solvents can often provide useful information regarding its homogeneity, D.P. and in some cases, by comparison with authentic specimens, its exact identity.

The movement of a sugar as a single component in at least three different solvents including acidic, basic and neutral systems, is a useful indication of homogeneity. While this often holds good for disaccharides it may not always hold for tri- and higher saccharides and exceptions have been reported. Thus BACON[13] found that two of the fructosylsucrose trisaccharides commonly produced in invertase–sucrose digests tended to move as a single discrete spot on paper chromatograms and were only separable on charcoal–celite columns. HATANAKA[74] has also reported that two galactosylsucrose tetrasaccharides could not be separated on paper chromatograms or

References p. 133.

charcoal columns; the presence of two sugars was only deduced by chemical means.

In general, disaccharides move at slower rates than their constituent monosaccharides. Exceptions to this rule are rhodymenabiose (3-O-β-D-xylanopyranosyl-D-xylose) which, in some solvents, moves in front of xylose[89] and sucrose which moves at the same rate as an aldohexose in many solvents. Whilst disaccharides rarely move at the same chromatographic rate as pentose and hexose monosaccharides, confusion can arise in the case of the heptoses which have small R_F values. This can often be avoided by the use of the appropriate spray reagents. Comparison of the movement of an unknown oligosaccharide with known oligosaccharides will generally give an indication of D.P. which must, however, be checked by other methods. In the case of the members of an homologous series, FRENCH AND WILD[67] have established that the relationship between the number of monosaccharide units per molecule and the logarithm of the partition function, $R_F/(1-R_F)$, is linear. The use of this empirical relationship will indicate whether the sugars present in a mixture are in fact the members of an homologous series.

It is often possible to establish the identity of an oligosaccharide, particularly a disaccharide, by comparing its movement with that of known sugars in a selection of solvents. Although this can be achieved by using published R_F or R_G values, it is preferable and safer wherever possible to compare the movement of the unknown sugar with authentic specimens on the same paper chromatograms. In general, however, the identification of an oligosaccharide should never be considered complete until a specimen of the sugar has been isolated and examined by chemical methods.

(c) *Spray reagents*

In conjunction with the rates of movement of sugars in various solvents, reactions with spray reagents can generally provide a reliable identification of most monosaccharides. With oligosaccharides, the sprays can contribute useful, and sometimes in the case of disaccharides conclusive evidence of structure.

The spray reagents are required to elucidate, if possible, the following points:

1. Location of the oligosaccharide on the paper chromatogram.
2. Presence or absence of a reducing unit.
3. Identity of the monosaccharide reducing unit.
4. The point of attachment of the O-glycosidic linkage to the reducing unit.
5. Position of other O-glycosidic linkages.
6. Identity of non-reducing monosaccharide units.

A large number of spray reagents which react with sugars are available. HOUGH[85] has reviewed in detail their reactions with monosaccharides. In Table 2 an attempt has been made to classify these reagents according to their ability to react with oligosaccharides and to provide evidence concerning the points listed above. Sensitivity has also been indicated on the basis of the amount of sugar on a chromatographic spot which will just allow the colour reaction in question to be observed clearly.

The most widely used technique for applying reagents is by spraying their

References p. 133.

TABLE 2

SPRAY REAGENTS

No.	Reagent	Sensitivity*	Reaction**	Reference
*A. Locating reducing and non-reducing oligosaccharides****				
1.	Silver nitrate-acetone/NaOH–ethanol	VHS	R and NR differentiated (not specific for carbohydrates)	142
2.	Periodate/*p*-anisidine hydrochloride	HS	R and NR give same reaction	40
3.	Periodate/potassium permanganate	HS	R and NR give same reaction	108
4.	Periodate/potassium permanganate/benzidine	MS	R and NR give same reaction	151
5.	Periodate/Schiff's reagent	MS	R and NR give same reaction	44
6.	Borate–phenol red	MS	R and NR give same reaction	81
7.	Vanillin–perchloric acid	HS	Reacts with most R and NR giving various colours (also reacts with phenols)	106
B. Reacting with specific sugar groups in oligosaccharides†				
8.	*p*-Anisidine hydrochloride (or phosphate)	VHS-HS	TR; With hydrochloride aldohexoses and 6-deoxyhexoses brown, ketohexoses yellow, aldo- and keto-pentoses pink, hexuronic acids pink	9, 47, 86
9.	3,5-Dinitrosalicylic acid–NaOH	HS	TR; brown	93
10.	Triphenyltetrazolium chloride (see No. 36)	HS	TR; red	32, 57, 143
11.	Aniline and diphenylamine–H_3PO_4 (see No. 37)	MS-LS	TR; various colours	19, 45, 135
12.	Phloroglucinol–HCl (or CCl_3CO_2H)	MS-LS	TR; various colours	35
13.	β-Naphthylamine–$Fe_2(SO_4)_3$–HCl	MS	TR; various colours	119
14.	α-Naphthylamine–CCl_3CO_2H	HS	TR; various colours	86
15.	*m*-Phenylenediamine–HCl	HS	TR; various colours	48
16.	Aniline hydrogen phthalate	VHS-HS	TR; various colours; weak reaction with ketoses	9, 121
17.	Aniline hydrogen oxalate	VHS-HS	TR; various colours; weak reaction with ketoses	86
18.	Aniline hydrogen phosphate	VHS-HS	TR; various colours; weak reaction with ketoses	65, 86
19.	Benzidine–acetic acid (or CCl_3CO_2H)	HS	TR; brown	16, 84
20.	Ninhydrin (for N-benzyl glycosylamine derivatives)	HS	TR; aldoses only, blue	31
21.	2,5-Diphenyl-3-*p*-styryl-phenyltetrazolium chloride	HS	TR; ketoses only, purple	14
22.	Urea–H_3PO_4 (or HCl)	VHS-HS	M: ketoses only, blue with H_3PO_4; black with HCl. Heptoses pink	86, 150 70

(continued on p. 122)

References p. 133.

TABLE 2 (*continued*)

No.	*Reagent*	*Sensitivity**	*Reaction***	*Reference*
23.	α-Naphthol–H_3PO_4	HS	M; ketoses blue	3
24.	Orcinol–HCl(or CCl_3CO_2H)	MS	M; ketoses only, red	86, 103
25.	Resorcinol–HCl (or CCl_3CO_2H)	MS	M; ketoses red	59, 86
26.	Naphthoresorcinol–HCl (or CCl_3CO_2H)	MS-LS	M; ketoses red, pentoses and hexuronic acids violet	59, 120
27.	Anthrone–H_3PO_4 and acetic acid	MS	M; ketoses only, purple-yellow	97
28.	Bromophenol blue	HS-MS	M; uronic acids	52
29.	Periodate/sodium nitroprusside/piperazine	MS	Deoxy-sugars blue	56
30.	Periodate/*p*-nitroaniline–HCl	MS	Deoxy-sugars deep yellow (not 6-deoxy derivatives)	56
31.	Quinine sulphate	HS	Sugar phosphates U.V. fluorescence	131
32.	Molybdate–perchloric acid/H_2S	MS-LS	M; sugar phosphates blue	72
33.	Ninhydrin	HS	M; amino sugars blue	29, 122, 124, 137
34.	Acetylacetone–NaOH/*p*-dimethylaminobenzaldehyde–HCl	HS	Amino sugars red, N-acetylhexosamine purple-violet	51, 134
35.	Periodate-glycol–thiobarbiturate	VHS	M(?); sialic acid and deoxy-sugars	144
	C. Indicating specific O-glycosidic linkages			
36.	Triphenyltetrazolium chloride	HS	1 → 2; no reaction if C_2 of reducing unit substituted	32, 57, 143
37.	Aniline and diphenylamine–H_3PO_4	MS-LS	1 → 4; deep blue if C_4 of reducing unit substituted (colours given by other reducing hexose disaccharides depend to some extent on linkage to reducing unit)	19, 45, 135
38.	Diazouracil	MS	"Sucrose linkage"; blue. Generally no reaction if fructose moiety is substituted	41, 130

* Approximate sensitivity: VHS < 5 μg; HS, 5–10 μg; MS, 10–20 μg; LS > 20 μg.

** R, reducing sugar; NR, non-reducing sugar; TR, terminal reducing unit; M, sugar present at any position in a chain.

*** These reagents will not function with highly substituted mono- and oligo-saccharides.

† Specificities may be determined in some cases by the strength of the associated acid and the degree of heating to which the chromatograms are subjected after spraying.

solutions on to the dried chromatogram. Spraying with certain compounds, *e.g.* benzidine, may be hazardous and therefore dipping the paper in the reagent may be a safer procedure. In this case the reagent must be dissolved in a solvent (*e.g.* acetone in the case of oligosaccharides) in which sugars are insoluble. A number of special

References p. 133.

dipping reagents have been developed by GORDON, THORNBURG AND WERUM[69].

Silver nitrate is probably the best locating spray. Although it suffers from the disadvantage that it is very unspecific and reacts with a number of other groups of compounds, *e.g.* phenols, it is highly sensitive and the sprayed papers, if washed with ammonia[142], are permanent records. The reagent, as published, also distinguishes between reducing and non-reducing sugars by the rate at which the coloured spots develop, the non-reducing sugars reacting more slowly. Provided sufficient non-reducing sugar is on the paper (5–10 μg or more) this difference may be accentuated by using half-strength silver nitrate solution. In this case non-reducing substances take 15–20 min to appear as compared with 1–2 min for reducing substances. Periodate sprays (reagent Nos. 2–5) are also useful for locating non-reducing substances, the initial periodate reaction depending on the presence of adjacent hydroxyl groups in the sugar molecule.

Reducing oligosaccharides can be located on paper by the use of a number of reagents, for example *p*-anisidine hydrochloride (No. 8), aniline hydrogen phthalate (No. 16) or benzylamine–ninhydrin (No. 20). In some instances (*e.g.* No. 8) an indication of the type of monosaccharide at the reducing end of an oligosaccharide chain may also be obtained. It should be noted, however, that strongly acidic reagents such as *p*-anisidine hydrochloride, will give positive reactions with non-reducing oligosaccharides which are acid labile, *e.g.* sucrose. Monosubstitution at C-2 or C-4 of the terminal reducing unit can often be detected using the triphenyltetrazolium chloride (No. 36) and aniline/diphenylamine (No. 37) reagents, respectively, and the so-called "sucrose linkage" can be identified with diazouracil (No. 38). In addition, reagents Nos. 8[9], 11, 16–18 give varying colour reactions, which to some extent appear to be related to the type of substitution at the reducing terminal unit. With this latter case, provided care is used in interpreting such results, and the colours so obtained are compared with those given by authentic specimens, much useful information can often be obtained.

The presence of ketose residues at any position in short oligosaccharide chains can be fairly easily demonstrated, *e.g.* with sprays Nos. 22–27. These reagents are strongly acidic and the colour reactions in all cases are almost certainly dependent on the prior conversion of the ketose, to a furfural derivative which then gives a coloured complex with the reagent. The acid may also have to hydrolyse glycosidic linkages before reaction can occur. When the acid used in the reagent is relatively weak, *e.g.* trichloroacetic acid, a positive reaction may only be obtained when the ketose unit is held in combination by an acid-labile linkage, such as in raffinose. When this linkage is not of the furanoside type, then the reagent should be made more acidic in order to detect the ketose. Thus leucrose (5-O-α-D-glucopyranosyl-D-fructopyranose) gives a positive reaction with sprays Nos. 25 and 26 in the presence of HCl, but not when trichloroacetic acid is used. The urea reagent (No. 22) only gives a typical ketose colour reaction with leucrose after prolonged heating[18]. Tests for combined ketose should, therefore, always include a spray reagent sufficiently acidic to react with these more stably linked oligosaccharides.

References p. 133.

Uronic acid units in any position in a chain can often be detected by the use of a suitable acid–base indicator such as bromophenol blue (No. 28). Oligosaccharides containing amino sugars may be located with ninhydrin (No. 33) and phosphates with the molybdate–perchloric acid reagent (No. 32). The existence of other non-reducing monosaccharide units with less reactive functional groups is almost impossible to prove unless the oligosaccharide is first hydrolysed. Similarly, it is very difficult to obtain information regarding the nature of the "internal" glycosidic bonds with spray reagents without resorting to partial hydrolysis of the oligosaccharides.

(d) Quantitative paper chromatography

Most of the methods available for the quantitative determination of the components of monosaccharide mixtures can conveniently be used for oligosaccharides. They may be divided into two main groups:

(i) where mixtures are resolved on chromatograms and the components eluted from the paper and then determined and

(ii) where the analytical reactions are actually carried out on the paper.

With the first group of methods the solution of the sugar mixture is normally streaked along the origin of the chromatogram which is then developed with a suitable solvent and dried. The positions of the bands of the separated components are then located by spraying marker strips which have been cut from both edges and from the centre of the chromatograms. The latter strip is important as the sugar bands may not be linear. The bands are then cut out, the sugars eluted, and portions of the eluates used for analysis. Elution may be achieved by a number of techniques including shaking the strips in tubes with water[132], extracting them in a reflux apparatus[58], or by elution in a chromatography tank. Using this quantitative method absolute values may be obtained by either (a) applying accurately measured volumes of sugar solutions to the paper (excluding the marker strips[105]) or (b) by adding a known weight of a "foreign" sugar to the solution under examination and determining this together with the other components[6]. In this latter instance, the volume of solution applied to the paper need not be measured accurately.

Numerous micro-methods, both volumetric and colorimetric, are available for the determination of the sugars in the eluates from the chromatographic strips. These include the use of the SOMOGYI reagent[136], sodium metaperiodate[79], phenol–H_2SO_4 reagent[54], benzidine[98], and anthrone[17,30,32,75,83,91].

With the second group of methods, which are colorimetric, the reactions are carried out on the paper chromatogram. Solutions to be analysed can be applied as spots to the paper and the developed chromatogram is sprayed with a suitable reagent which produces coloured or fluorescent derivatives with the sugars. The spots are then cut out of the chromatogram and the colours eluted and measured in a spectrophotometer. The reagents used for monosaccharides include *p*-anisidine hydrochloride[126], aniline hydrogen phthalate[12,149], and *o*-aminobiphenyl[125], and some of these could, no doubt, be adapted for use with oligosaccharides. This general

References p. 133.

technique is superior to those using marker strips in that there is never any doubt regarding the exact positions of the sugars on the chromatograms, and only very small volumes of a sugar solution are required for analysis. The main disadvantage of the technique is that standard compounds, in measured amounts, often have to be run on the same chromatogram as the sugar solutions which are being analysed. The application of an accurately measured volume of solution to the paper or the addition of a "foreign" sugar is also necessary with this technique.

Related to this latter group of methods are less common procedures, such as spraying the chromatogram with a suitable reagent, then photographing the chromatogram and measuring, photometrically, the intensity of the sugar spots on the negative, or measuring the spot intensity directly on the chromatogram[34,111,112].

3. PAPER ELECTROPHORESIS

In addition to paper chromatography, the related technique of paper electrophoresis can provide useful confirmatory evidence regarding the structure of an oligosaccharide. Wherever possible, electrophoresis should be used in conjunction with paper chromatography and the identity of an oligosaccharide checked by comparing its electrophoretic rate of movement with that of an authentic specimen.

The literature up to 1958 on this subject includes reviews by FOSTER[62] and MICHL[118] and a description of a simple paper electrophoresis apparatus by FOSTER[60]. Most observations on the electrophoretic behaviour of oligosaccharides have been carried out using an alkaline borate buffer. For example, FOSTER[61] has recorded the rates of movement, relative to D-glucose (M_G values), of various glucopyranosyl disaccharides and PRIDHAM[128] has examined the mobilities of the raffinose family of oligosaccharides and a series of $1 \rightarrow 4$ β-linked D-mannose oligosaccharides (D.P. 2–5). In the case of the raffinose series the behaviour is highly characteristic of the group, the mobilities increasing in the order: raffinose $<$ stachyose $<$ verbascose $<$ tetragalactosylsucrose. With the mannose oligosaccharides, however, the more usual behaviour of decreasing rate of movement with increasing molecular weight was observed.

Paper electrophoresis, using a borate buffer, has also been useful for the characterisation of the various fructosylsucrose derivatives which can be obtained from sucrose by enzymic transglycosylation reactions[4,13,71,127] and for trisaccharides of D-glucose, resulting from the partial hydrolysis of an *Aspergillus niger* polyglucan[27] and glycogen[123,152,153]. M_G values for some oligosaccharides are given in Table 3.

Other electrolyte systems which have been used for this technique include sodium arsenite, basic lead acetate, sodium hydroxide[65], sodium tungstate, sodium metavanadate and ammonium or sodium molybdate[37,66]. With the exception of ammonium molybdate, however, these systems have mainly been used with monosaccharides and their derivatives and the behaviour of oligosaccharides has been largely ignored. A sodium molybdate–sulphuric acid mixture (pH 5) has been used by BOURNE, HUTSON AND WEIGEL[37], to distinguish between disaccharide alcohols

References p. 133.

TABLE 3

M_G VALUES FOR SOME OLIGOSACCHARIDES IN BORATE BUFFER

Oligosaccharide	*Structure**	M_G	*Reference*
α,α-Trehalose	α-G 1-1 α-G	0.19	63
α,β-Trehalose	α-G 1-1 β-G	0.23	63
β,β-Trehalose	β-G 1-1 β-G	0.19–0.20	63
Sophorose	β-G 1-2 G	0.24	38
Nigerose	α-G 1-3 G	0.69	61
Laminaribiose	β-G 1-3 G	0.69	61
Maltose	α-G 1-4 G	0.32	61
Cellobiose	β-G 1-4 G	0.28	61
Isomaltose	α-G 1-6 G	0.69	61
Gentiobiose	β-G 1-6 G	0.75	61
Maltotriose	α-G 1-4 α-G 1-4 G	0.33	153
Isomaltotriose	α-G 1-6 α-G 1-6 G	0.57	91
Panose	α-G 1-6 α-G 1-4 G	0.33	153
—	α-G 1-4 α-G 1-3 G	0.62	22
—	α-G 1-3 α-G 1-4 G	0.32	22
Lactose	β-Gal 1-4 G	0.38	61
Melibiose	α-Gal 1-6 G	0.80	61
—	β-G 1-1 Fru	0.74	38
Sucrose	α-G 1-2 β-Fru	0.10, 0.17	38, 63
Turanose	α-G 1-3 Fru	0.69	38
Maltulose	α-G 1-4 Fru	0.63	38
Leucrose	α-G 1-5 Fru	0.56	38
Isomaltulose	α-G 1-6 Fru	0.60	38
Maltotriulose	α-G 1-4 α-G 1-4 Fru	0.35	11
Glucosylleucrose	α-G 1-6 α-G 1-5 Fru	0.44	91
Isomaltotriulose	α-G 1-6 α-G 1-6 Fru	0.31	11
Melezitose	α-G 1-2 β-Fru 3-1 α-G	0.22	36
Mannobiose	β-Man 1-4 Man	0.69	128
Mannotriose	β-Man 1-4 β-Man 1-4 Man	0.67	128
Mannotetraose	β-Man 1-4 β-Man 1 $_2$-4 Man	0.63	128
Mannopentaose	β-Man 1-4 β-Man 1 $_3$-4 Man	0.59	128
Raffinose	α-Gal 1-6 α-G 1-2 β-Fru	0.31	128
Stachyose	α-Gal 1-6 α-Gal 1-6 α-G 1-2 β-Fru	0.35	128
Verbascose	α-Gal 1-6 α-Gal 1 $_2$-6 α-G 1-2 β-Fru	0.43	128
Tetragalactosylsucrose	α-Gal 1-6 α-Gal 1 $_3$-6 α-G 1-2 β-Fru	0.50	128

* Fru = D-fructose; G = D-glucose; Gal = D-galactose; Man = D-mannose.

possessing various glycosidic linkages. The mobilities were in the following order: $1 \rightarrow 3 < 1 \rightarrow 4 < 1 \rightarrow 6$ and $1 \rightarrow 2$; the corresponding reducing disaccharides had very low mobilities. (Reducing disaccharides can, of course, be readily reduced to the corresponding alcohols with potassium borohydride.[39]) The same workers[38] have also studied the mobilities of glucopyranosyl-fructoses in molybdate and other buffer systems.

An estimate of the D.P. value for an oligosaccharide may be achieved by paper electrophoresis using sodium bisulphite solution as the electrolyte[64]. The mobilities of oligosaccharides under these conditions are dependent on the molecular weight and in the main are not influenced by the stereochemistry or the types of glycosidic linkages present in the molecule. Similar estimates may also be made by preparing the N-benzylglycosylamine derivatives of reducing oligosaccharides and examining their electrophoretic mobilities under strongly acidic conditions[24].

Silver nitrate (spray No. 1; Table 2) is suitable for locating sugars and sugar

References p. 133.

alcohols on paper electrophoretograms. The electrolytes on the paper may slow down its rate of reaction to some extent. FRAHN AND MILLS[65] discuss the effect of ions on spray reagents and describe suitable modifications. BOURNE, FOSTER AND GRANT[36] claim that fibre glass sheet supports are superior to Whatman No. 3 paper in that non-reducing sugars can be detected more readily, absorption processes are reduced and the separation of sugars with similar absolute mobilities is facilitated.

4. COLUMN CHROMATOGRAPHY

After tentative identification of an oligosaccharide by paper chromatography it is desirable to isolate sufficient of the pure compound for absolute identification by chemical and physical methods. Prior to the application of preparative chromatography to the carbohydrate field it was virtually impossible to isolate oligosaccharides from complex mixtures unless they were present as major components and, in the past, such sugars were only regarded as pure and adequately characterised when they had been prepared in crystalline form. However, during recent years many oligosaccharides have been isolated only as chromatographically homogeneous, freeze-dried, ash-free powders or syrups. Such preparations are now generally regarded as sufficiently pure for structural investigations.

The separation of oligosaccharides on columns can be effected by three main methods: these are: (1) partition chromatography on cellulose or celite, (2) adsorption chromatography on charcoal and (3) ion-exchange resin chromatography. In addition, it should be mentioned here that small amounts of oligosaccharides can generally be obtained by preparative paper chromatography. This is achieved by streaking the sugar solution along the starting line of the paper, with appropriate marker spots and processing as described in the analytical section (p. 124). By using a thick paper, *e.g.* Whatman No. 3 MM, workable amounts of sugar can readily be separated. A second processing is often required to obtain a chromatographically pure compound.

(*a*) *Partition on cellulose*

This method was first used by HOUGH, JONES AND WADMAN[86] for the resolution of monosaccharide mixtures. It is, however, readily applicable to the separation of mixtures of oligosaccharides, although in this case the procedure is somewhat slower. In theory a particular solvent system should, for a given mixture of sugars, give the same pattern of separation on a paper partition chromatogram as on a cellulose column. However, it is probably more difficult to achieve the same degree of separation on the cellulose column as on paper.

The apparatus required for cellulose column chromatography has been described by LEDERER AND LEDERER[107]. The important practical points are concerned with the selection of a solvent system, packing and loading of the column and collection and analysis of the eluted fractions.

Solvent systems. The system used should be selected by paper chromatographic studies with the sugar mixture which is to be resolved, using various solvent systems.

References p. 133.

For the separation of monosaccharides alcohol (*e.g.* *n*-butanol or isopropanol)–water mixtures have been used and in general simple binary systems are preferable to the more complex mixtures often used for paper chromatography.

Packing and loading the column. This is most important, particularly if undue tailing of sugars with consequent poor resolution of closely moving components in a mixture is to be avoided. Columns may be packed dry or wet. Dry packing is described by HOUGH, JONES AND WADMAN[86]. Wet packing is probably easier. A simple technique is to prepare the cellulose, by thorough mixing in a blendor, as a slurry in a solvent such as aqueous acetone (50 %). The slurry is poured into the glass column (empty or partially filled with aqueous acetone) and the powder allowed to settle under gravity and the solvent to drain to within approximately one inch of the surface of the cellulose. It is then washed successively with mixtures of acetone–water, acetone–resolving solvent and finally resolving solvent. A much slower packing technique which, however, produces columns with high powers of resolution, is to transfer the slurry to the column, filled with solvent, using a bulb pipette with a fine tip. In this way the formation of small lumps of cellulose powder is avoided and a very evenly packed column is obtained[21]. The packing can be checked by allowing suitable dyes to pass down the column with the resolving solvent[86]. If the dye does not move in a reasonably compact band, the column should be repacked. The dyes may also be used as markers, so that unnecessary fractions need not be collected and examined before the sugars start to come off the column.

Collection and analysis of eluted fractions. Suitable fraction collectors are available from many manufacturers and need not be described here. The composition of the eluted fractions may be determined qualitatively by paper chromatography or quantitatively by various analytical procedures. Anthrone is one of the most useful reagents for quantitative measurements of the total sugars in the fractions. This reagent has been used for the determination of free and combined hexoses[32,91,140] and amino sugars[83], and for free pentoses[17], uronic acids[75] and sugar phosphates[39]. There is little doubt that the method could also be adopted for oligosaccharides containing the latter three sugars.

There is little danger of losing monosaccharides or oligosaccharides of low D.P. by adsorption on to the cellulose. The main disadvantages of these columns are: (1) large numbers of small fractions must be collected and analysed for component sugars (*cf.* charcoal), (2) oligosaccharides may take a long time to move through the column and (3) the resolution of higher oligosaccharides may be poor. Cellulose columns will, however, often resolve mixtures which are not resolved easily by other methods of column chromatography. The poor resolution of the higher oligosaccharides may be overcome by developing the cellulose column until di- or tri-saccharides have been eluted. The column can then be extracted, sectioned and the sugars finally extracted from each section. MALPRESS AND HYTTEN[115] describe the use of this technique for the fractionation of the sugar mixture obtained from human milk. THOMA, WRIGHT AND FRENCH[139] have operated cellulose columns at elevated temperatures, and have been able to fractionate maltodextrins up to D.P. 18.

References p. 133.

A commercial improvement of the cellulose column is the wound paper column (made by L. K. B. Producta, Sweden). Packing problems are avoided and relatively large amounts of sugars can be resolved with precision comparable to that obtained by ordinary paper chromatography. Sugar mixtures should first be treated with a small part of cellulose to remove any substances that are likely to be irreversibly absorbed on to the paper coil. Full details of the operation of this column are available from the manufacturers.

(b) Partition on celite

LEMIEUX, BISHOP AND PELLETIER[109] replaced cellulose with celite for the partition chromatography of monosaccharides and their derivatives. They claim that columns are superior from the point of view of speed, ease of packing, and of operation and product purity. BACON[14] in an investigation of closely related plant trisaccharides has used small celite columns for purifying the fractions isolated from a charcoal column. The relative merits of celite and cellulose columns should be carefully considered before attempting the fractionation of an oligosaccharide mixture by partition chromatography.

(c) Adsorption on charcoal

The separation of oligosaccharides on columns of charcoal was first described by WHISTLER AND DURSO[147]. The method depends on their differential adsorption on to the charcoal followed by fractional elution with aqueous solvents.

Adsorbent. Generally the charcoal is mixed with one to two volumes of celite to aid flow. JERMYN[95] recommends cellulose instead of celite; this gives faster flow rates and also avoids contamination of the eluates with colloidal silica. For most work ordinary grades of charcoal "suitable for decolourising" are satisfactory. The charcoal–celite mixture may be cleaned by washing firstly with HCl then absolute ethanol[28, 110], or with citrate buffer[146], and finally with distilled water. The washed charcoal is then dried at 80°. Columns of the mixture are best packed as a wet slurry; details of the packing of columns are given by WHELAN, BAILEY AND ROBERTS[146], BARKER, BOURNE AND THEANDER[28], and LINDBERG AND WICKBERG[110].

Approximately 25 g of charcoal should be used per g of sugar placed on the column. Although some workers have applied suction or pressure to the column to aid flow rates, elaborate arrangements seem to be unnecessary. With most grades of charcoal satisfactory flow rates can be obtained by having the eluent reservoir from 6–8 ft. above the column.

Simple solvent fractionation. With this technique the sugars are adsorbed on to the top of the column and, after removal of monosaccharides with water, oligosaccharides are eluted with water containing an alcohol, normally ethanol. Disaccharides are generally desorbed from the charcoal with 4–6 % aqueous ethanol, trisaccharides with 8–10 % and so on up to heptaoses with approximately 28–30 % aqueous ethanol. This method is ideal for fractionating an homologous series of oligosaccharides. With the higher members of such series there tends to be some overlap of the sugars in the

References p. 133.

fractions. This can be avoided if after removal of a major fraction, the column is well washed with an ethanol–water mixture of a slightly higher ethanol concentration which will remove any traces of the already eluted compound, but will not elute the next higher oligosaccharide. It is not necessary to collect large numbers of samples, and, provided the column has been carefully packed, high flow rates (up to several drops per second) may be used. The technique is thus suitable for large scale fractionations. The method, for example, has been used for the preparation of members of the following homologous series; maltodextrins[146], isomaltodextrins[20] and xylan oligosaccharides[148].

Normally oligosaccharides up to a D.P. value of 6–7 can be eluted from the charcoal column; the hexaoses and heptaoses normally come off with 30 % aqueous ethanol but with higher alcohol concentrations elution ceases. Using a charcoal of low adsorptive capacity, BAILEY AND CLARKE[20] were able to extend the fractionation of the isomaltodextrins up to the octaose and isomaltose was eluted from the column with only 1–2 % aqueous ethanol.

Charcoal fractionation can often be speeded up by using the modification described by ANDREWS, HOUGH AND POWELL[7], in which the charcoal is packed as a short, wide column in a sintered glass Buchner funnel. This method is particularly useful for purifying individual oligosaccharides or for removing a very high concentration of a mono- or a disaccharide from a solution containing trace amounts of other sugars. We have found the method to be particularly useful for freeing commercial maltose from traces of glucose and maltotriose. Quite large quantities of maltose (10–15 g on a column 8 × 13 cm of charcoal) can be dealt with if half the column is packed and then the remainder of the charcoal stirred (1–2 h) with an aqueous solution of the maltose and this slurry finally poured on to the top of the packed charcoal.

Gradient elution. It is evident that the simple fractionation technique is unsuitable for the resolution of a mixture containing, for example, several di- or trisaccharides. In this case modifications of the method are necessary. The simplest still uses aqueous ethanol but employs gradient elution as described by BACON AND BELL[15] and by BARKER *et al.*[25,28]. Briefly, instead of attempting complete elution of, for example, a disaccharide in a single aqueous ethanol fraction, the column is washed with solvent of gradually increasing ethanol concentration over the range 0–8 % ethanol. Many small fractions must be collected and analysed for their constituent sugars. The method depends on the fact that all oligosaccharides of the same D.P. are not eluted by identical alcohol concentrations and thus some degree of separation may be achieved. In addition to the results reported by the above authors, JERMYN[95] gives a detailed study of the method and describes the use of a number of other aqueous solvents as eluents. BACON[14] describes the use of the technique for the resolution of closely related trisaccharides isolated from plant material. Apart from complete separations the method is often of value for the partial purification of sugars prior to partition chromatography on cellulose or celite. The technique has been used to resolve mixtures of sugars which moved as a single spot on paper chromatograms[13].

Fractionation on borate- and molybdate-treated charcoal columns. The ability of

References p. 133.

some sugars to form borate complexes was used by BARKER, BOURNE AND THEANDER[28], in another modification of charcoal chromatography in which sodium borate was added to the column and eluent. Disaccharides which are capable of forming borate complexes are eluted at much lower ethanol concentrations than the normal 4–6 % aqueous ethanol which desorbs the free disaccharides. Thus these authors found that, using gradient elution, 1 → 3 and 1 → 6 linked glucose disaccharides were eluted with 0.8–1.2 % aqueous ethanol but the corresponding 1 → 4 and 1 → 2 linked compounds required 4–6 % aqueous ethanol to remove them from the borate–carbon column. (Details of the removal of borate ions from the eluates are given on p. 115). With complex mixtures of oligosaccharides the method is generally suitable only for the isolation of small amounts of the pure compounds. In certain cases, however, *e.g.* with a mixture of isomaltose and maltose, the method is suitable for isolations on a larger scale.

BARKER *et al.*[23] have also shown that a molybdate-treated charcoal–celite column with aqueous ethanol containing molybdate as eluent gives useful separations. In general, the sugars which are eluted most readily as molybdate complexes are those (*e.g.* maltose) which do not form fast running borate complexes.

Separation of sugars as furanosides on charcoal columns. A further modification of charcoal chromatography developed by BARKER, BOURNE AND O'MANT[26] utilises the fact that treatment of disaccharides with methanolic HCl at room temperature converts some, but not all, to their methyl furanosides. After adsorption of the sugars on a standard charcoal–celite column unreacted disaccharides are eluted with 4–6 % aqueous ethanol, whereas the furanosides require 10–20 % aqueous ethanol for elution. Thus maltose can easily be separated from nigerose and cellobiose from laminaribiose. The furanosides may readily be reconverted to the disaccharides by treatment with dilute acid. The method has wide application and could be applied on a large scale.

Most of the methods described above have been developed with a view to the complete separation of all of the sugars in a complex oligosaccharide mixture. However, it is should be remembered that where only one component of a mixture is required, enzymic destruction of the other constituents may simplify the separation. Thus treatment of a maltose–isomaltose mixture with maltase or glucamylase preparations converts it into a more easily resolvable mixture of isomaltose and glucose.

(*d*) *Ion-exchange chromatography*

Ion-exchange resins may be used either for the fractionation of neutral sugars (as ionised complexes) or, more commonly, for the separation of sugars possessing ionised groups (*e.g.* sugar phosphates, sugar carboxylic acids and amino sugars), from one another and from neutral components. The technique for preparing and using ion-exchange resins together with descriptions of the main type of resins have been adequately described elsewhere[46,92].

Fractionation of neutral sugars. The principle of this method is the formation by sugars, of charged complexes with ions such as borate. The complexes may be ad-

References p. 133.

sorbed on to a resin, *e.g.* Dowex-1 (borate form) and then selectively eluted with borate buffers.

The method has been applied to the resolution of monosaccharides (*e.g.* KHYM, ZILL AND COHN[102]) but does not appear to have been used extensively for resolving oligosaccharides. It is probable that in future other forms, such as molybdate, of anion exchange resins, will be used effectively for the separation of sugars.

JONES, WALL AND PITTET[100] have reported a good, rapid separation of D-glucose, sucrose and raffinose on a column of Dowex-50 W (Li^+ form, using water as the eluent); resin size appeared to be important. Extensions of this method may prove to be of value particularly for the fractionation of oligosaccharides with D.P. values greater than 2–3 as elution proceeds in order of decreasing molecular size. Ion-exchange resins are also of value for the separation of neutral sugars from those possessing ionizable functions such as carboxyl, phosphate, or amino groups. The method is comparatively simple; the sugar solution is passed down a column of a suitable ion-exchange resin (cation, anion or mixed resin exchanger, depending on the components in the sugar mixture). Neutral sugars may be washed from the column with water leaving the other compounds adsorbed on the resin. The latter may then either be completely or fractionally eluted with an appropriate eluent. MACHELL[113], points out that resins used to adsorb sugar acids should be in the carbonate form, otherwise neutral sugars may be destroyed.

Fractionation of acidic and basic sugars. In a number of separations of acidic or basic oligosaccharides ion-exchange chromatography has proved advantageous and a few examples are given below:

(i) *Sugar carboxylic acids.* Fractionations of these sugars are generally carried out on columns of an anion exchange resin in the formate or acetate form using aqueous formic or acetic acids as eluents. DERUNGS AND DEUEL[53], for example, using Dowex-3 (formate form) fractionated the oligogalacturonate series obtained by the hydrolysis of pectic acid. WEISSMANN *et al.*[145] (using Dowex-1, formate form) have also resolved the oligoglucuronates produced by the enzymic hydrolysis of hyaluronic acid.

Oligosaccharides containing one hexuronic acid residue are often obtained by the hydrolysis of plant gums and hemicelluloses. In the past a mixture of such sugars has generally been seperated by fractional precipitation of their salts or by cellulose chromatography. Resolution on an ion-exchange column may in many instances offer a better method for separating these compounds. ASPINALL, HIRST AND MATHESON[10], for example, have recently fractionated a mixture of acidic oligosaccharides obtained by the partial hydrolysis of *Khaya grandifolia* gum, on a column of Amberlite IRA-400 (acetate form; aqueous acetic acid eluent). Similarly ADAMS[1] has used Dowex-1 (acetate form; aqueous formic acid eluent) to fractionate hexuronic acid-containing oligosaccharides obtained from Spruce hemicellulose.

(ii) *Sugar phosphates.* Separations in this field have been concerned mainly with the resolution of monosaccharide and, occasionally, disaccharide phosphates. A review of early work on the fractionation of these compounds on resins is given by BENSON[33]. KHYM, ZILL AND COHN[101, 102] found that good separations of sugar phos-

References p. 133.

phates, adsorbed on Dowex-1 (Cl^- form), required the presence of small amounts of borate in the eluent (NH_4OH–NH_4Cl solution). Several workers[30,82,104] have also fractionated sugar phosphates on Dowex-1 (Cl^- or formate form), by gradient elution with formic acid or HCl solutions. The methods used by these authors should prove satisfactory for phosphorylated oligosaccharides.

(iii) *Amino sugars.* The fractionation of amino sugars can be effected on a cation exchange resin of the Dowex-50 or Zeo-Karb 225 type. An example of such a fractionation is given by HOROWITZ, ROSEMAN AND BLUMENTHAL[83] who resolved the homologous series of D-glucosamine oligosaccharides obtained by the partial hydrolysis of chitin on a column of Dowex-50 (H^+ form) by gradient elution with dilute HCl.

Apart from the collection of large numbers of fractions the main problem with ion-exchange resin chromatography lies in removing the eluting agent from the sugars. Procedures for the removal of HCl, formic acid and borate ions are described in a previous section (p. 115). In preliminary paper chromatographic analysis of the eluates the relatively low concentrations of acidic ions present in many eluting systems are not likely to cause gross interference. Cations should, however, be removed from borate eluates with a strong cation exchange resin. Spots of the acidic solutions should not be dried on the papers at high temperatures in order to avoid possible reversion[87] or destruction of labile sugars. Formic acid present in column effluents does not interfere with anthrone[30] in the measurement of sugar concentrations. With this reagent a correction may be made for interference by HCl[30] or the acid first removed[83].

The preceding review has illustrated the great value of the many varied chromatographic techniques which are available for the study of the chemistry and biochemistry of oligosaccharides. Much work has been done on these special analytical and preparative procedures but there is, of course, still room for improvements. For example, column chromatography, which is at present tedious, would benefit from more "automation", perhaps on similar lines to that which has been applied to the fractionation of amino acid mixtures. More spray reagents with highly specific reactions for the various molecular groups and linkages found in these sugars are desirable and the possible use of cellulose ion-exchangers for fractionation, on a wide scale, should be borne in mind.

REFERENCES

1 G. A. ADAMS, *Can. J. Chem.*, 37 (1959) 29.
2 R. A. AITKEN, B. P. EDDY, M. INGRAM AND C. WEURMAN, *Biochem. J.*, 64 (1956) 63.
3 N. D. ALBON AND D. GROSS, *Analyst*, 75 (1950) 454.
4 P. J. ALLEN AND J. S. D. BACON, *Biochem. J.*, 63 (1956) 200.
5 P. ANDREWS, D. H. BALL AND J. K. N. JONES, *J. Chem. Soc.*, (1953) 4090.
6 P. ANDREWS, L. HOUGH AND J. K. N. JONES, *J. Chem. Soc.*, (1952) 2744.
7 P. ANDREWS, L. HOUGH AND D. B. POWELL, *Chem. & Ind. (London)*, (1956) 658.
8 A. R. ARCHIBALD AND D. J. MANNERS, *Biochem. J.*, 73 (1959) 292.
9 K. ASO AND F. YAMAUCHI, *Tohoku J. Agr. Research*, 5 (1955) 305.
10 G. O. ASPINALL, E. L. HIRST AND N. K. MATHESON, *J. Chem. Soc.*, (1956) 989.
11 G. AVIGAD, *Biochem. J.*, 73 (1959) 587.
12 S. BAAR, *Biochem. J.*, 58 (1954) 175.
13 J. S. D. BACON, *Biochem. J.*, 57 (1954) 320.
14 J. S. D. BACON, *Biochem. J.*, 73 (1959) 507.

[15] J. S. D. BACON AND D. J. BELL, *J. Chem. Soc.*, (1953) 2528.
[16] J. S. D. BACON AND J. EDELMAN, *Biochem. J.*, 48 (1951) 114.
[17] R. W. BAILEY, *Biochem. J.*, 68 (1958) 669.
[18] R. W. BAILEY, *Nature*, 181 (1958) 836.
[19] R. W. BAILEY AND E. J. BOURNE, *J. Chromatog.*, 4 (1960) 206.
[20] R. W. BAILEY AND R. T. J. CLARKE, *Biochem. J.*, 72 (1959) 49.
[21] S. A. BARKER, personal communication.
[22] S. A. BARKER, E. J. BOURNE AND J. G. FLEETWOOD, *J. Chem. Soc.*, (1957) 4805.
[23] S. A. BARKER, E. J. BOURNE, A. B. FOSTER AND R. B. WARD, *Nature*, 179 (1957) 262.
[24] S. A. BARKER, E. J. BOURNE, P. M. GRANT AND M. STACEY, *Nature*, 177 (1956) 1125.
[25] S. A. BARKER, E. J. BOURNE, G. C. HEWITT AND M. STACEY, *J. Chem. Soc.*, (1955) 3734.
[26] S. A. BARKER, E. J. BOURNE AND D. M. O'MANT, *Chem. & Ind. (London)*, (1955) 425.
[27] S. A. BARKER, E. J. BOURNE AND M. STACEY, *J. Chem. Soc.*, (1953) 3084.
[28] S. A. BARKER, E. J. BOURNE AND O. THEANDER, *J. Chem. Soc.*, (1955) 4276.
[29] S. A. BARKER, A. B. FOSTER, M. STACEY AND J. M. WEBBER, *J. Chem. Soc.*, (1958) 2218.
[30] G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 449, 459.
[31] R. J. BAYLY AND E. J. BOURNE, *Nature*, 171 (1953) 385.
[32] D. J. BELL, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. II, Springer, Verlag, Berlin, 1955, p. 1.
[33] A. A. BENSON, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. II, Springer Verlag, Berlin 1955, p. 113.
[34] A. BEVENUE AND K. T. WILLIAMS, *J. Chromatog.*, 2 (1959) 199.
[35] E. BORENFREUND AND Z. DISCHE, *Arch. Biochem. Biophys.*, 67 (1957) 239.
[36] E. J. BOURNE, A. B. FOSTER AND P. M. GRANT, *J. Chem. Soc.*, (1956) 4311.
[37] E. J. BOURNE, D. H. HUTSON AND H. WEIGEL, *Chem. & Ind. (London)*, (1959) 1047.
[38] E. J. BOURNE, D. H. HUTSON AND H. WEIGEL, *Chem. & Ind. (London)*, (1960) 1111.
[39] P. D. BRAGG AND L. HOUGH, *J. Chem. Soc.*, (1957) 4347.
[40] P. D. BRAGG AND L. HOUGH, *J. Chem. Soc.*, (1958) 4050.
[41] H. J. BREUER AND J. S. D. BACON, *Biochem. J.*, 66 (1957) 462.
[42] D. R. BRIGGS, E. F. GARNER, R. MONTGOMERY AND F. SMITH, *Anal. Chem.*, 28 (1956) 1333.
[43] J. L. BUCHAN AND R. I. SAVAGE, *Analyst*, 77 (1952) 401.
[44] J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162.
[45] H. W. BUSTON AND A. H. KHAN, *Biochim. Biophys. Acta*, 19 (1956) 564.
[46] C. CALMON AND T. R. E. KRESSMAN, *Ion Exchangers in Organic and Biochemistry*, Interscience Publ. Inc., New York, 1957.
[47] J. CERBULIS, *Anal. Chem.*, 27 (1955) 1400.
[48] E. CHARGAFF, C. LEVINE AND C. GREEN, *J. Biol. Chem.*, 175 (1948) 67.
[49] R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
[50] R. H. CÔTÉ AND W. J. T. MORGAN, *Nature*, 178 (1956) 1171.
[51] M. J. CRUMPTON, *Biochem. J.*, 72 (1959) 479.
[52] A. L. DEMAIN AND H. J. PHAFF, *Arch. Biochem. Biophys.*, 51 (1954) 114.
[53] R. DERUNGS AND H. DEUEL, *Helv. Chim. Acta*, 37 (1954) 657.
[54] M. DUBOIS, K. GILLIES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Nature*, 168 (1951) 167.
[55] W. F. DUDMAN AND J. F. WILKINSON, *Biochem. J.*, 62 (1956) 289.
[56] J. T. EDWARDS AND D. M. WALDRON, *J. Chem. Soc.*, (1952) 3631.
[57] D. S. FEINGOLD, G. AVIGAD AND S. HESTRIN, *Biochem. J.*, 64 (1956) 351.
[58] E. A. FLOOD, E. L. HIRST AND J. K. N. JONES, *J. Chem. Soc.*, (1948) 1679.
[59] W. G. C. FORSYTHE, *Nature*, 161 (1948) 239.
[60] A. B. FOSTER, *Chem. & Ind. (London)*, (1952) 1050.
[61] A. B. FOSTER, *J. Chem. Soc.*, (1953) 982.
[62] A. B. FOSTER, *Advances in Carbohydrate Chem.*, 12 (1957) 81.
[63] A. B. FOSTER AND M. STACEY, *J. Chem. Soc.*, (1955) 1778.
[64] J. L. FRAHN AND J. A. MILLS, *Chem. & Ind. (London)*, (1956) 578.
[65] J. L. FRAHN AND J. A. MILLS, *Chem. & Ind. (London)*, (1956) 1137.
[66] J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65.
[67] D. FRENCH AND G. M. WILD, *J. Am. Chem. Soc.*, 75 (1953) 2612.
[68] G. GIOVONNOZZI-SERMANNI, *Nature*, 177 (1956) 586.
[69] H. T. GORDON, W. THORNBURG AND L. N. WERUM, *Anal. Chem.*, 28 (1956) 849.
[70] F. L. GREENE, *Anal. Chem.*, 30 (1958) 1164.
[71] D. GROSS, *Nature*, 173 (1954) 487.
[72] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
[73] F. E. G. HARRAP, *Nature*, 182 (1958) 876.
[74] S. HATANAKA, *Arch. Biochem. Biophys.*, 82 (1959) 188.
[75] J. R. HELBERT AND K. D. BROWN, *Anal. Chem.*, 28 (1956) 1098.

[76] R. W. HENDERSON, R. K. MORTON AND W. K. RAWLINSON, *Biochem. J.*, 72 (1959) 340.
[77] S. HESTRIN AND G. AVIGAD, *Biochem. J.*, 69 (1958) 388.
[78] R. HEYWORTH AND J. S. D. BACON, *Biochem. J.*, 66 (1957) 41.
[79] E. L. HIRST AND J. K. N. JONES, *J. Chem. Soc.*, (1949) 1659.
[80] E. L. HIRST AND J. K. N. JONES, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. II, Springer Verl., Berlin, 1955, p. 275.
[81] D. J. D. HOCKENHULL, *Nature*, 171 (1953) 982.
[82] B. L. HORECKER, P. Z. SMYRNIOTIS AND J. E. SEEGMILLER, *J. Biol. Chem.*, 193 (1951) 383.
[83] S. T. HOROWITZ, S. ROSEMAN AND H. J. BLUMENTHAL, *J. Am. Chem. Soc.*, 79 (1957) 5046.
[84] R. H. HORROCKS AND G. B. MANNING, *Lancet*, 256 (1949) 1042.
[85] L. HOUGH, in D. GLICK, *Methods of Biochemical Analysis*, Vol. I, Interscience Publ. Inc., New York, 1954, p. 205.
[86] L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702.
[87] L. HOUGH AND J. B. PRIDHAM, *Chem. & Ind. (London)*, (1957) 1178.
[88] L. HOUGH AND J. B. PRIDHAM, *Biochem. J.*, 73 (1959) 550.
[89] B. H. HOWARD, *Biochem. J.*, 67 (1957) 643.
[90] R. C. HUGHES AND W. J. WHELAN, *Chem. & Ind. (London)*, (1958) 884.
[91] D. H. HUTSON, unpublished results.
[92] *Ion Exchange Resins*, British Drug Houses, Ltd., Poole, England.
[93] A. JEANES, C. S. WISE AND R. J. DIMLER, *Anal. Chem.*, 23 (1951) 415.
[94] M. A. JERMYN, *Australian J. Biol. Sci.*, 8 (1955) 541.
[95] M. A. JERMYN, *Australian J. Chem.*, 10 (1957) 55.
[96] M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402.
[97] R. JOHANSON, *Nature*, 172 (1953) 956.
[98] J. K. N. JONES AND J. B. PRIDHAM, *Biochem. J.*, 58 (1954) 288.
[99] J. K. N. JONES AND W. W. REID, *J. Chem. Soc.*, (1954) 1361.
[100] J. K. N. JONES, R. A. WALL AND A. O. PITTET, *Chem. & Ind. (London)*, (1959) 1196.
[101] J. X. KHYM AND W. E. COHN, *J. Am. Chem. Soc.*, 75 (1953) 1153.
[102] J. X. KHYM, L. P. ZILL AND W. E. COHN, in C. CALMON AND T. R. E. KRESSMAN, *Ion Exchangers in Organic and Biochemistry*, Interscience Publ. Inc., New York, 1957, p. 392.
[103] R. KLEVESTRAND AND A. NORDAL, *Acta Chem. Scand.*, 4 (1950) 1320.
[104] V. KLYBAS, M. SCHRAMM AND E. RACKER, *Arch. Biochem. Biophys.*, 80 (1959) 229.
[105] R. A. LAIDLAW AND S. G. REID, *J. Sci. Food Agr.*, 3 (1952) 19.
[106] M. G. LAMBOU, *Anal. Chem.*, 29 (1957) 1449.
[107] E. LEDERER AND M. LEDERER, *Chromatography*, 2nd Ed., Elsevier, Amsterdam, 1957.
[108] R. U. LEMIEUX AND H. F. BAUER, *Anal. Chem.*, 26 (1954) 920.
[109] R. U. LEMIEUX, C. T. BISHOP AND G. E. PELLETIER, *Can. J. Chem.*, 34 (1956) 1365.
[110] B. LINDBERG AND B. WICKBERG, *Acta Chem. Scand.*, 8 (1954) 569.
[111] R. M. MCCREADY AND E. A. MCCOMB, *Anal. Chem.*, 26 (1954) 1645.
[112] E. F. MCFARREN, K. BRAND AND H. R. RUTKOWSKI, *Anal. Chem.*, 23 (1951) 1146.
[113] G. MACHELL, *J. Chem. Soc.*, (1957) 3389.
[114] A. M. MACLEOD AND H. MCCORQUODALE, *Nature*, 182 (1958) 815.
[115] F. H. MALPRESS AND F. E. HYTTEN, *Biochem. J.*, 68 (1958) 708.
[116] F. H. MALPRESS AND A. B. MORRISON, *Nature*, 164 (1949) 963.
[117] R. J. MARTINEZ, J. B. WOLFE AND H. I. NAKADA, *J. Biol. Chem.*, 234 (1959) 2236.
[118] H. MICHL, *J. Chromatog.*, 1 (1958) 93; *Chromatographic Reviews*, Vol. 1, Elsevier, Amsterdam, 1959, p. 11.
[119] L. NOVELLIE, *Nature*, 166 (1950) 745.
[120] S. M. PARTRIDGE, *Nature*, 158 (1946) 270.
[121] S. M. PARTRIDGE, *Biochem. J.*, (1948) 238; S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
[122] W. J. PAYNE, R. J. KIEBER AND C. JEFFRIES, *Arch. Biochem. Biophys.*, 52 (1954) 1.
[123] S. PEAT, W. J. WHELAN AND T. E. EDWARDS, *J. Chem. Soc.*, (1955) 355.
[124] H. R. PERKINS, *Biochem. J.*, 74 (1960) 182.
[125] C. V. PIPER AND L. J. BERNARDIN, *Tappi*, 41 (1958) 16.
[126] J. B. PRIDHAM, *Anal. Chem.*, 28 (1956) 1967.
[127] J. B. PRIDHAM, *Biochem. J.*, 76 (1960) 13.
[128] J. B. PRIDHAM, unpublished results.
[129] E. W. PUTMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods of Enzymology*, Vol. III, Academic Press Inc., New York, 1957, p. 62.
[130] H. W. RAYBIN, *J. Am. Chem. Soc.*, 55 (1933) 2603.
[131] E. S. ROREM, *Nature*, 183 (1959) 1739.
[132] A. ROUDIER, *Mémorial des Services Chimiques de l'État*, 36 (1951) 161; publ. by Laboratoire Central des Services Chimiques de L'État, Paris.
[133] V. C. RUNECKLES AND G. KROTKOV, *Arch. Biochem. Biophys.*, 80 (1959) 94.

134 M. R. J. SALTON, *Biochim. Biophys. Acta*, 34 (1959) 308.
135 S. SCHWIMMER AND A. BEVENUE, *Science*, 123 (1956) 543.
136 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61.
137 P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373.
138 J. A. THOMA AND D. FRENCH, *Anal. Chem.*, 29 (1957) 1645.
139 J. A. THOMA, H. B. WRIGHT AND D. FRENCH, *Arch. Biochem. Biophys.*, 85 (1959) 452.
140 W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 50 (1952) 298.
141 W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 62 (1956) 177.
142 W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
143 K. WALLENFELS, *Naturwiss.*, 37 (1950) 491.
144 F. L. WARREN, *Nature*, 186 (1960) 237.
145 B. WEISSMANN, K. MEYER, P. SAMPSON AND A. LINKER, *J. Biol. Chem.*, 208 (1954) 417.
146 W. J. WHELAN, J. M. BAILEY AND P. J. P. ROBERTS, *J. Chem. Soc.*, (1953) 1293.
147 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677.
148 R. L. WHISTLER AND C. C. TU, *J. Am. Chem. Soc.*, 74 (1952) 3609.
149 C. M. WILSON, *Anal. Chem.*, 31 (1959) 1199.
150 C. S. WISE, R. J. DIMLER, H. A. DAVIS AND C. E. RIST, *Anal. Chem.*, 27 (1955) 33.
151 M. L. WOLFROM AND J. B. MILLER, *Anal. Chem.*, 28 (1956) 1037.
152 M. L. WOLFROM AND A. THOMPSON, *J. Am. Chem. Soc.*, 78 (1956) 4182.
153 M. L. WOLFROM AND A. THOMPSON, *J. Am. Chem. Soc.*, 79 (1957) 4212.

A 14

[*Reprinted from the Proceedings of the Biochemical Society*, 11–12 *January* 1962.
Biochem. J., 1962, Vol. 82, No. 3, 44P.]

The Biosynthesis of Galactosylsucrose Derivatives

By E. J. Bourne, J. B. Pridham and Mary W. Walter. (*Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey*)

Galactosylsucrose derivatives are widely distributed in the plant kingdom (French, 1954; Courtois, 1959) although little is known about the biosynthesis or metabolism of these oligosaccharides. Members of the 'raffinose family' of oligosaccharides are formed in some seeds as the tissues mature and they are then rapidly metabolized when the seeds germinate (e.g. Pridham, 1958).

Incubation of α-D-galactose 1-phosphate with [^{14}C]sucrose in the presence of ATP, UTP and an enzyme preparation from *Vicia faba* seeds yielded an oligosaccharide which was indistinguishable from raffinose on paper chromatograms and electrophoretograms. This sugar was further characterized by hydrolysis with N-H_2SO_4 which yielded labelled glucose and fructose and inactive galactose, and by treatment with yeast invertase which produced melibiose and fructose both labelled. The melibiose was identified by paper chromatography and electrophoresis and by reduction to melibi-itol with potassium borohydride. Labelled glucitol was produced by acidic hydrolysis of the melibi-itol.

Preliminary studies also suggest that *V. faba* α-galactosidase can transfer α-galactopyranosyl residues to sucrose acceptor molecules (cf. Anagnostopoulos, Courtois & Petek, 1955). The biosynthesis of galactosylsucrose derivatives *in vivo* was discussed with reference to the pathways described.

One of us (M.W.W.) is indebted to the Agricultural Research Council for a studentship.

Anagnostopolous, C., Courtois, J. E. & Petek, F. (1955). *Arch. Sci. biol., St Pétersb.*, **39**, 631.

Courtois, J. E. (1959). In *Proc. IVth int. Congr. Biochem.* vol. I, p. 140. Ed. by Wolfrom, M. L. London: Pergamon Press.

French, D. (1954). *Advanc. Carbohyd. Chem.* **9**, 149.

Pridham, J. B. (1958). *Nature, Lond.*, **182**, 1687.

A 15

REPRINTED FROM
ADVANCES IN CARBOHYDRATE CHEMISTRY - VOLUME 17

ACADEMIC PRESS INC., NEW YORK, N. Y.

OLIGOSACCHARIDES

By R. W. Bailey

Plant Chemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand

AND

J. B. Pridham

Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey, England

I. Introduction

Since the last review in this Series on the subject of oligosaccharides as such,[1] a vast amount of literature on its various aspects has been published, and many new compounds have been isolated. The aim of the present article is to consider one aspect of oligosaccharide chemistry, the determination of structure, and to provide up-to-date Tables of the oligosaccharides which have been isolated and characterized. As a comprehensive account of all of the methods used for structural analysis is obviously impossible in the space available, the authors have concentrated on those methods, developed in the past ten years, which require only small amounts of oligosaccharide.

Following the introduction of paper-chromatographic techniques, some fifteen years ago, there has been a steady change in the methods used for determining oligosaccharide structure. Many compounds are now only

(1) W. L. Evans, D. D. Reynolds, and E. A. Talley, *Advances in Carbohydrate Chem.*, **6**, 27 (1951); see also J. Staněk, M. Černý, and J. Pacák, "Oligosacharidy," Československé Academie Věd, Prague, 1962.

isolated as chromatographically pure, freeze-dried powders or sirups, and probably more reliance is placed on such indirect methods as periodate oxidation. Many of the techniques now available have been largely made possible by paper chromatography. In spite of this, the desirability of crystallizing new oligosaccharides should not be overlooked, and the preparation of crystalline derivatives should always be undertaken.

In defining the structure of an oligosaccharide, the following points must be established, preferably by more than one method: (1) degree of polymerization; (2) whether reducing or nonreducing; (3) identity and sequence of constituent monosaccharides; (4) position and sequence of the glycosidic links; (5) configuration of the glycosidic links; and (6) size of the monosaccharide rings. The methods described in the following Sections should enable all of these features to be ascertained.

II. Determination of Oligosaccharide Structure

1. *Paper Chromatography and Ionophoresis*

An extensive description of the use of these invaluable procedures need not be given here, as detailed accounts may be found elsewhere.[2] The following is a summary of the main uses of paper chromatography and ionophoresis in structural studies.

Rate of movement on chromatograms is generally a good indication of degree of polymerization. There is, however, at least one disaccharide, 3-*O*-β-D-xylopyranosyl-D-xylose,[3] which has been reported to move faster than its constituent monosaccharide, suggesting caution in determining degree of polymerization (D.P.) from rate of movement. Many spray-reagents will indicate whether an oligosaccharide is reducing or not, and will enable the reducing glycose residue to be defined at least as an aldose or a ketose. For a detailed description of the use of these spray reagents with oligosaccharides, see Bailey and Pridham.[2] Two spray-reagents can sometimes indicate the position of the glycosidic link on the reducing glycose residue. First, failure of a reducing oligosaccharide to react with triphenyltetrazolium chloride indicates a glycosidic link on C-2 of a reducing aldose or C-1 of a reducing ketose.[4] Second, reducing aldohexoses having a glycosidic link at C-4 of the reducing unit give a characteristic blue color with diphenylamine–aniline.[5]

(2) L. Hough, *Methods of Biochem. Anal.*, **1**, 205 (1954); A. B. Foster, *Advances in Carbohydrate Chem.*, **12**, 81 (1957); E. Lederer and M. Lederer, "Chromatography," Elsevier Publishing Co., Amsterdam, 2nd Edition, 1957; R. W. Bailey and J. B. Pridham, *Chromatog. Revs.*, **4**, 114 (1962).

(3) B. H. Howard, *Biochem. J.*, **67**, 643 (1957).

(4) K. Wallenfels, *Naturwissenschaften*, **37**, 491 (1950); G. Avigad, R. Zelikson, and S. Hestrin, *Biochem. J.*, **80**, 57 (1961).

(5) S. Schwimmer and A. Bevenue, *Science*, **123**, 543 (1956).

The position of the glycosidic link at the reducing end of an oligosaccharide may also be determined by paper ionophoresis. If a reducing oligosaccharide is converted to its alditol (e.g., by reduction with potassium borohydride[6]) migration in molybdate buffer is determined by the position of the glycosidic link on the alcohol residue.[7] A distinction can, therefore, be made between aldohexose oligosaccharides having glycosidic links on C-3, C-4, and C-2 or C-6 of the reducing unit. Comparison of the migration of reducing, fructosyl oligosaccharides on ionophoretograms run in molybdate, borate, arsenite, and sodium hydroxide, respectively, differentiates compounds having glycosidic links on any of the carbon atoms of the fructosyl unit.[8] The mobility of oligosaccharides under these conditions is dependent on structure rather than on molecular size, and the results obtained are probably more reliable than those from paper chromatography.

If an isolated oligosaccharide is believed to be of known structure, and authentic specimens of the known compound, and its isomers, are available, detailed comparison on paper chromatograms and ionophoretograms will often suffice to identify it. The identification should, if possible, be confirmed by comparison of the properties of crystalline derivatives. If the compound is of unknown structure, or authentic specimens are lacking, definite identity should never be assigned solely on the basis of the chromatographic properties of the compound.

2. *Acid Hydrolysis*

The particular value of acid hydrolysis is that, used in conjunction with paper chromatography, much evidence may be obtained from very small amounts of compound. As the identification of monosaccharides by paper chromatography is well established, the use of this technique in conjunction with total, acid hydrolysis provides a ready means for identifying, both qualitatively and quantitatively, the monosaccharide components of an oligosaccharide. Acid stability of different glycosidic links differs, and total hydrolysis may require from one to four hours at 100° in *N* acid; even more vigorous conditions may be required when amino sugar or uronic acid residues are present. Very rapid hydrolysis in dilute acid, 0.1–0.2 *N*, generally indicates the presence of a nonreducing, furanosyl unit.

With tri- and oligo-saccharides, partial acid hydrolysis offers a useful means of determining the nature of at least some of the links. Much milder hydrolytic conditions are required and these must be determined experimentally for each compound studied. The disaccharide fragments of

(6) M. L. Wolfrom and H. B. Wood, *J. Am. Chem. Soc.*, **73**, 2933 (1951); P. D. Bragg and L. Hough, *J. Chem. Soc.*, 4347 (1957).

(7) E. J. Bourne, D. H. Hutson, and H. Weigel, *Chem. & Ind.* (London), 1047 (1959).

(8) E. J. Bourne, D. H. Hutson, and H. Weigel, *Chem. & Ind.* (London), 1111 (1960).

an oligosaccharide are often known compounds, authentic specimens of which are available; and many of the fragments may, therefore, be reasonably well identified by paper techniques. In this way, the identity, but not necessarily the position, of the links can often be established. Because of the differing acid-stability of glycosidic links, it is often not possible to obtain a disaccharide fragment containing an acid-labile link from an oligosaccharide containing several links much more stable to acid. The rapid hydrolysis of sucrose under mild conditions (0.1–0.2 *N* acid, 15–20 min. at 100°) permits the preferential cleavage of the sucrose residues present in many tri- and oligo-saccharides (see Table IIIc), thus providing more-easily identifiable fragments. To avoid acid reversion, with the consequent production of artefactual oligosaccharides, partial hydrolyses should be conducted in dilute solutions containing less than 1% of sugar.

Hydrolyses of oligosaccharide alditols (produced by borohydride or Raney nickel–hydrogen reduction[6]) provide further structural information. Identification of the surviving, reducing monosaccharide in the total hydrolyzate of a reduced, heterogeneous disaccharide will establish which of the two monosaccharides was the original reducing residue. Partial hydrolysis of a reduced trisaccharide, followed by paper ionophoresis, in molybdate buffer, of the disaccharide alditol fragment will often provide evidence identifying the link attached to the original, reducing glycose residue. Identification of the other reducing disaccharide fragment will then provide a good indication of total structure.[8a] Two disaccharide alditols in the partial hydrolyzate of a tri- or oligo-saccharide alditol is evidence for the presence of a "branched" structure involving the reducing monosaccharide residue.[9] Partial, acid hydrolysis of the oligosaccharide and its alditol has also been extended to tetra- and oligo-saccharides. Thus, the use of these methods was of considerable value in the identification of two unknown D-glucose tetrasaccharides, each of which contained one (1→3) and two (1→6) links, when only 30 mg. of the mixed compounds was available.[10] In general, results obtained from partial acid hydrolyses should be regarded as only confirmatory evidence of structure.

A useful method for measuring the D.P. of a reducing oligosaccharide involves a comparison of the reducing power of total hydrolyzates of the oligosaccharide and its alditol.[11] As originally developed, the method is only suitable for homogeneous oligosaccharides; by the use of a different

(8a) M. L. Wolfrom, A. Thompson, and T. T. Galkowski, *J. Am. Chem. Soc.*, **73**, 4093 (1951).

(9) R. W. Bailey, S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey, *J. Chem. Soc.*, 1895 (1958).

(10) R. W. Bailey, D. H. Huston, and H. Weigel, *Biochem. J.*, **80**, 514 (1961).

(11) S. Peat, W. J. Whelan, and J. G. Roberts, *J. Chem. Soc.*, 2258 (1956).

means of measuring total reducing power, Timell[12] has extended the method to heterogeneous compounds.

Acid hydrolysis, as described, gives a largely uncontrolled degradation. Stepwise degradation of oligosaccharides can often be achieved by the selective hydrolysis of their osazones on an acid-type ion-exchange resin.[13] This procedure was used by Howard[3] to confirm the structure proposed for a D-xylose trisaccharide.

3. *Periodate Oxidation*

a. General Principles.—Periodate oxidation is now probably the most widely used technique for investigating oligosaccharide structure. It has,

TABLE I

Oxidation of R′—CH(OH)—R″ *with the Periodate Ion*

R′	*Periodate consumption, moles/mole*	*Products*
—CHO	1	HCO_2H, R″CHO
—CH_2OH	1	HCHO, R″CHO
R | —CH(OH)	1	R″CHO, RCHO
R | CH(OH) | —CH(OH)	2	HCO_2H, R″CHO, RCHO

to some extent, replaced methylation analysis, for, although both methods give essentially the same information regarding ring sizes and substitution patterns, periodate oxidation is advantageous in view of its simplicity and the small amounts of oligosaccharides required. The quantitative cleavage of 1,2-glycols by periodic acid was discovered in 1928,[14] and Bobbitt[15] has reviewed the development of the method, including most of the theoretical and practical aspects of the subject up to 1955.

The most important oxidations which can be achieved with periodate are summarized in Table I. In addition, α-hydroxy ketones, α-amino alde-

(12) T. E. Timell, *Svensk Papperstidn.*, **63**, 668 (1960).

(13) P. A. Finan and P. S. O'Colla, *Chem. & Ind.* (London), 1387 (1955).

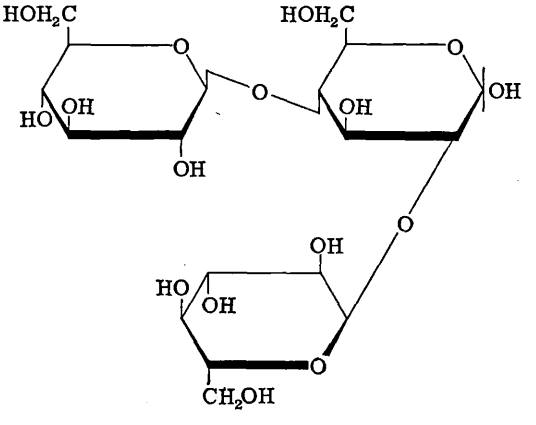
(14) L. Malaprade, *Bull. soc. chim. France*, **43**, 683 (1928); *Compt. rend.*, **186**, 382 (1928).

(15) J. M. Bobbitt, *Advances in Carbohydrate Chem.*, **11**, 1 (1956).

hydes, 2-amino alcohols, 1,2-diketones, and groups (such as malonaldehyde) which possess active hydrogen atoms are oxidized. The last reaction is responsible for "overoxidation"; it will be discussed later.

Full experimental details for the measurement of periodate uptake and the production of formic acid, formaldehyde, and carbon dioxide are given in many of the publications referred to in this Section. A spectrophotometric method for periodate determination,[16] modified for oligosaccharides,[17] is suitable for small-scale oxidations; it may replace the classical arsenite–iodometric method. Good micromethods for measurement of formaldehyde are also now available.[18]

b. Oxidation of Hexose- and Pentose-containing Oligosaccharides.—As most oligosaccharides contain simple hexose or pentose residues, the periodate oxidation of these compounds has been the most widely studied. Provided that "overoxidation" is avoided, such oxidation of an oligosaccharide, in terms of Table I, means the consumption of 1 mole of periodate per molar proportion of 1,2-diol, and the liberation of 1 mole of formic acid per molar proportion of 1,2,3-triol and of 1 mole of formaldehyde per mole of 1,2-diol carrying a primary alcohol group. If the constituent monosaccharide residues of the oligosaccharide are known, the molar ratio of the above three factors can be used to test the possible structures and eliminate many of them. Often, with the help of other evidence, conclusive identification may be obtained of the structure, in-



(1)

(16) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(17) G. O. Aspinall and R. J. Ferrier, *Chem. & Ind.* (London), 1216 (1957).

(18) J. F. O'Dea and R. A. Gibbons, *Biochem. J.*, **55**, 580 (1953); L. Hough, D. B. Powell, and B. M. Woods, *J. Chem. Soc.*, 4799 (1956); M. W. Rees, *Biochem. J.*, **68**, 118 (1958).

sofar as ring size and the position and sequence of glycosidic links is concerned. For example, branched trisaccharide[9] (1), on treatment with unbuffered sodium metaperiodate, consumed 3.9 moles of periodate per mole and produced 1.75 moles of formic acid. Its alditol, obtained by borohydride reduction,[6] consumed 5.02 moles of periodate and produced 1.92 and 0.85 moles of formic acid and formaldehyde, respectively, per mole. These results are readily correlated with the structure proposed, using the facts given in Table I. With trisaccharide (1) no cleavage of the reducing D-glucose residue, and hence no "overoxidation," can occur. To avoid such "overoxidation" with linear oligosaccharides, it is probably better to work with the oligosaccharide alditol[19] or methyl glycoside.[19]

With some oligosaccharides, periodate studies may be complicated by different rates of oxidation of the constituent monosaccharides, by hydrolysis, and by "overoxidation." In such cases, the periodate consumption and yields of products are strongly influenced by the reaction conditions, and the interpretation of results obtained should, therefore, be undertaken with great care.

(i) *Steric factors.*—The periodate oxidation of stachyose [*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside] can also be effected without complications and was used for showing that the D-galactose–D-glucose link is not (1→4) as originally believed, but (1→6).[20] With this sugar, however, the different monosaccharide residues present are oxidized at different rates, although this does not affect the final results (periodate consumption, 7 moles per mole; formic acid production, 3 moles per mole), provided that sufficient reaction time is allowed.

In this connection, it has long been known that 1,2-glycols having their hydroxyl groups locked in the true (antiparallel) *trans* position are resistant to periodate oxidation.[21] The difference in oxidation rates of the *cis* and *trans* isomers is particularly well illustrated by studies with 1,4-anhydro-L-erythritol and 1,4-anhydro-L-threitol, the former (*cis*) being oxidized more rapidly than the latter.[22] In the presence of a limited proportion of periodate, the D-glucosyl unit of sucrose is attacked more readily than the D-fructosyl unit, because the *trans*-1,2-glycol group in the latter

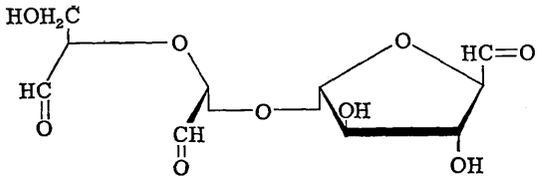
(19) M. L. Wolfrom, A. Thompson, A. N. O'Neil, and T. T. Galkowski, *J. Am. Chem. Soc.*, **74**, 1062 (1952); R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, 3536 (1957).

(20) H. Hérrissey, A. Wickstrøm, and J. E. Courtois, *Bull. soc. chim. biol.*, **34**, 856 (1952).

(21) C. C. Price and M. Knell, *J. Am. Chem. Soc.*, **64**, 552 (1942); F. Smith, *J. Chem. Soc.*, 633 (1944); R. J. Dimler, H. A. Davis, and G. E. Hilbert, *J. Am. Chem. Soc.*, **68**, 1377 (1946); B. H. Alexander, R. J. Dimler, and C. L. Mehltretter, *ibid.*, **73**, 4658 (1951).

(22) H. Klosterman and F. Smith, *J. Am. Chem. Soc.*, **74**, 5336 (1952).

is less favorably oriented.[23] The fact that D-galactopyranosyl rings, containing *cis*-1,2-glycol groups, are more readily oxidized than either D-glucopyranosyl or D-fructofuranosyl rings was used for obtaining sucrose from stachyose and raffinose by purely chemical means.[24] The oligosaccharides (1 mole) were treated with 3 and 2 moles of periodate, respectively, in ethanolic solution,[25] and sucrose was obtained from the resulting polyaldehyde by hydrolysis with sodium carbonate. The rate of the periodate oxidation of α-D-glucopyranosyl α-D-galactofuranoside has also been studied.[26] Long oxidation (800 min.) in 0.015 M sodium metaperiodate resulted in the consumption of only 3.7 moles of periodate per mole, with the production per mole of 1.45 moles and 0.83 mole of formic acid and formaldehyde, respectively. One mole of periodate per mole was consumed immediately, and the remainder during 260 min., presumably giving the intermediate trialdehyde (2). The authors suggested[26] that the subsequent decrease in the oxidation rate was attributable to the relative stability, to oxidation, of the trans-1,2-glycol grouping in (2).

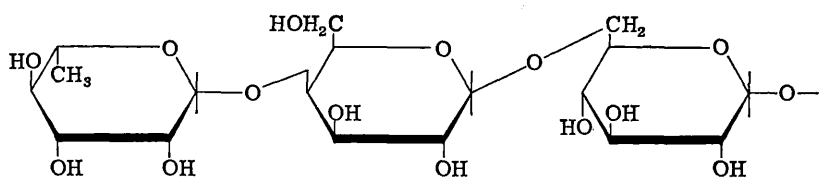


(2)

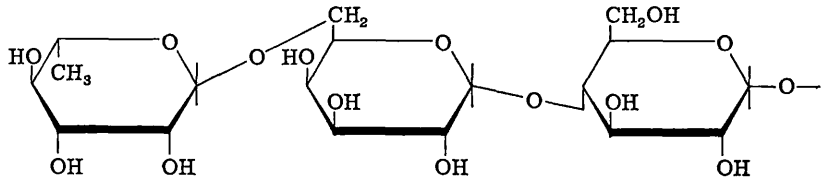
With hexoses, except for their derived alditols, formaldehyde is produced from a glycosidically bound, nonreducing, furanosyl residue, but not from a similar pyranosyl residue. In the periodate oxidation of oligosaccharides composed of monosaccharide residues commonly occurring in the pyranoid form, the possibility of liberation of formaldehyde should always be checked. Thus, with α-D-glucopyranosyl α-D-galactofuranoside,[26] the detection of nearly one mole of formaldehyde per mole, liberated early in the oxidation, was important evidence that the D-galactose residue occurs in the (unexpected) furanoid form.

The rate of production of formic acid by periodate from triol groups has been used in determining the structure of the glycosidically bound trisaccharide solanose.[27] Oxidation and other evidence suggested that the sugar moiety is either trisaccharide (3) or (4).

(23) A. K. Mitra and A. S. Perlin, *Can. J. Chem.*, **37**, 2047 (1959).
(24) A. K. Mitra and A. S. Perlin, *Can. J. Chem.*, **35**, 1079 (1957).
(25) P. F. Fleury, J. E. Courtois, and A. Bieder, *Bull. soc. chim. France*, **19**, 118 (1952).
(26) E. J. Bourne, J. Hartigan, and H. Weigel, *J. Chem. Soc.*, 1088 (1961).
(27) L. H. Briggs and L. C. Vining, *J. Chem. Soc.*, 2809 (1953).



(3)



(4)

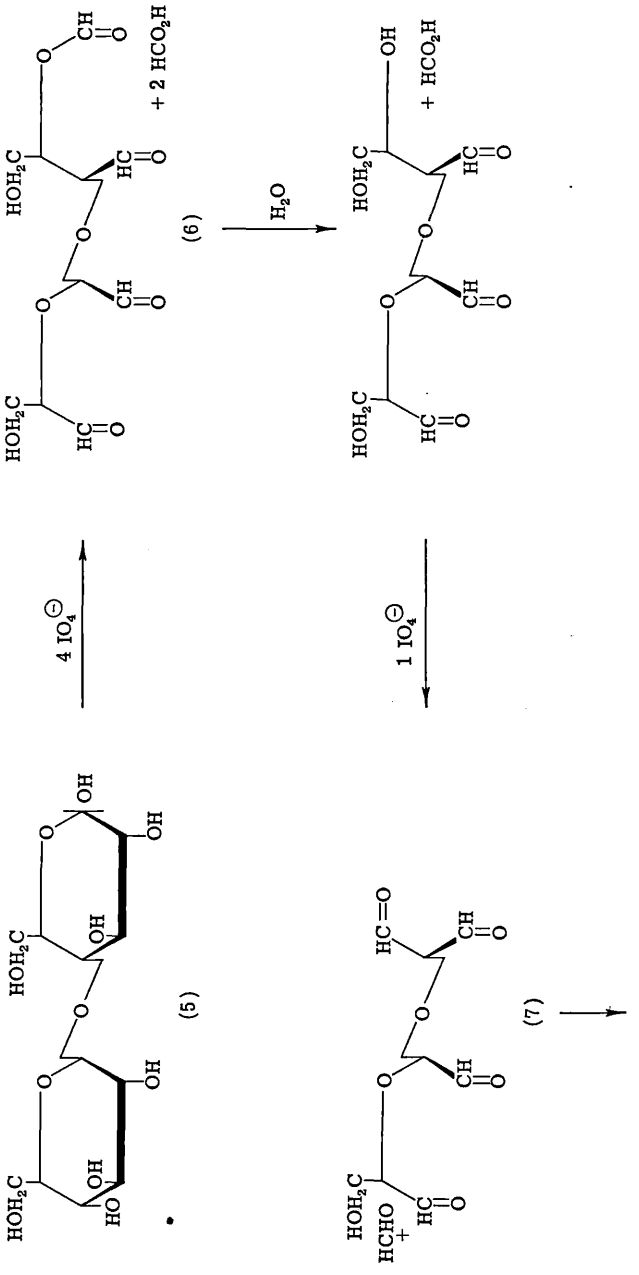
One mole of the glycoside consumed 5 moles of periodate and produced 1 mole of formic acid rapidly, followed by the slow release of a second mole of formic acid. This suggested that formula (3), with a *cis-trans* and a *trans-trans* triol group, is the correct structure, the former being oxidized more readily than the latter. Trisaccharide (4) has two *cis-trans* triol groups, which would be oxidized at *similar* rates. The same authors[27] showed that one mole of amygdalin consumes 4 moles of periodate and produces 2 moles of formic acid, with *no* rapid, initial production of acid; this is consistent with the presence of two *trans-trans* triol groups in this gentiobioside.

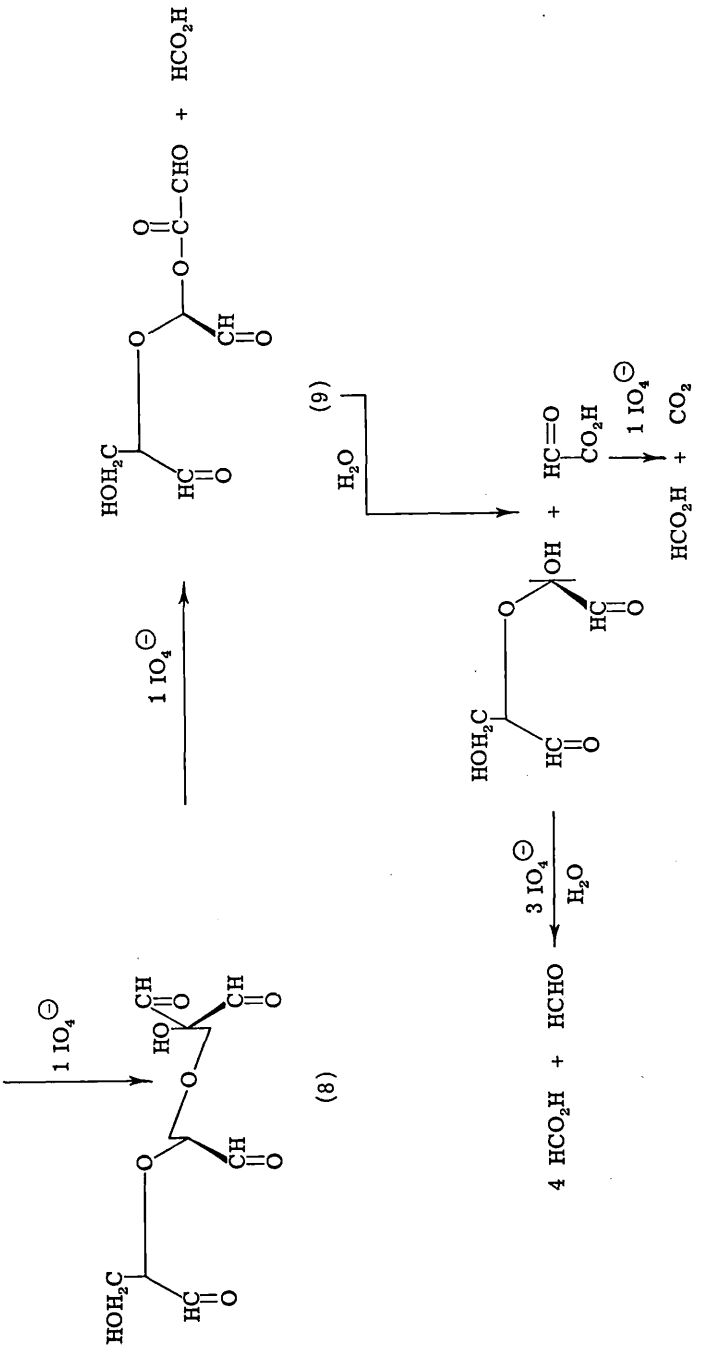
Large substituent groups also affect the oxidation of mono-[28] and oligosaccharides. With disaccharides, the large reducing end-group appears to hinder, sterically, the oxidation of the nonreducing unit. This is particularly noticeable when very dilute (0.4 m*M*) solutions of periodate are used; the disaccharides, and their alditols and aldobionic acids, behave in the same way.[29] Clancy,[30] for example, has shown that, in 7 hours, one mole of sophoritol (2-*O*-β-D-glucopyranosyl-D-glucitol) consumes 2.8 moles of periodate (corresponding to oxidation of the glucitol end-group) when treated with 0.4 m*M* periodate, but 5 moles when treated with 40 m*M* periodate. In each case, 1 mole of formaldehyde per mole is produced. This selective-oxidation technique has been employed to identify the gly-

(28) E. F. Garner, I. T. Goldstein, R. Montgomery, and F. Smith, *J. Am. Chem. Soc.*, **80**, 1206 (1958).

(29) M. J. Clancy and W. J. Whelan, *Chem. & Ind.* (London), 673 (1959).

(30) M. J. Clancy, *J. Chem. Soc.*, 4213 (1960).


+ 2 HCO2H
(6)
H2O
+ HCO2H
4 IO4⊖
1 IO4⊖
(5)
(7)
HCHO


1 IO4⊖
(8)
1 IO4⊖
(9)
H2O
3 IO4⊖
H2O
1 IO4⊖
4 HCO2H + HCHO
HCO2H + CO2

cosidic link on the reducing glycose residue of oligosaccharides.[29] It may be used with reducing oligosaccharides; or, they can be first converted to aldobionic acids or alditols, preferably the latter. Oxidation of the (1→4)-, (1→5)-, or (1→6)-linked acids under these conditions produces glyoxylic acid, which is stable to periodate oxidation and can be readily detected colorimetrically with phenylhydrazine and hydrogen peroxide. As (1→2)- and (1→3)-linked acids do not produce glyoxylic acid, the procedure is particularly useful for distinguishing between (1→3) and (1→4) links. With laminaribiitol, and cellobiitol, yield of periodate and consumption of product are the same, but the oxidation rates differ; cellobiitol reacts much more rapidly than laminaribiitol. Isomaltitol is oxidized faster than either cellobiitol or laminaribiitol, with a higher consumption of periodate. Comparison of the rates of oxidation of alditols in 0.4 m*M* periodate gives, therefore, a useful distinction between (1→3)-, (1→4)-, and (1→6)-linked disaccharides.[29]

(ii) *"Overoxidation."*—At high temperatures or periodate concentrations, the oxidative specificity of the reagent is lost, and "overoxidation" can occur. With some oligosaccharides, overoxidation can take place under mild conditions of reaction, particularly in alkaline solutions. Thus, (1→2)-, (1→3)-, and (1→4)-linked aldohexopyranose saccharides can be completely oxidized to formaldehyde, formic acid, and carbon dioxide under mild conditions, whereas the corresponding (1→5)- and (1→6)-linked compounds are relatively resistant to overoxidation.[31]

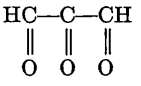
Overoxidation occurs mainly when oxidation of the 1,2-glycol groupings in the reducing end-group produces a malonaldehyde derivative [for example, (7)] possessing an activated hydrogen atom. The main path is then by way of a substituted hydroxymalonaldehyde[32] [for example, (8)] which is oxidized to a glyoxylyl ester [for example, (9)], whose hydrolysis exposes the nonreducing unit to complete oxidation. This sequence is explained with reference to cellobiose (5), whose oxidation has been studied by Head and Hughes.[31] When treated with unbuffered sodium metaperiodate at 20° in the dark, one mole of this sugar consumes 11 moles of periodate and produces 9, 2, and 1 moles of formic acid, formaldehyde, and carbon dioxide, respectively. The periodate consumption is in two stages, the first being a rapid (1 hour) uptake of 4 moles per mole, with the production of 2 moles of formic acid and a formyl ester derivative (6). After hydrolysis of this ester, the remaining periodate (7 moles

(31) F. S. H. Head and G. Hughes, *J. Chem. Soc.*, 603 (1954); C. F. Huebner, S. R. Ames, and E. C. Bubl, *J. Am. Chem. Soc.*, **68**, 1621 (1946); L. Hough and M. B. Perry, *Chem. & Ind.* (London), 768 (1956); F. S. H. Head and G. Hughes, *J. Chem. Soc.*, 2046 (1952); F. S. H. Head, *J. Textile Inst., Trans.*, **44**, 209 (1953).

(32) J. C. P. Schwarz and M. MacDougall, *J. Chem. Soc.*, 3065 (1956).

per mole) is consumed slowly (40 days) in the second stage; a very slow oxidation of the liberated formic acid and formaldehyde constitutes a third stage of the reaction requiring several months for completion. As well as temperature and concentration of periodate, pH strongly influences overoxidation. In weakly acid solutions (pH 3.5–5.0), the periodate consumption may be diminished, as the intermediary formyl and glyoxylyl esters are not readily hydrolyzed under these conditions.[33] At higher or lower pH values, however, these esters are hydrolyzed, and further oxidation can occur.

The sequence (5–9) is probably not the entire explanation of the mechanism of overoxidation. Thus, when cellobiitol was oxidized at 20° in 0.015 *M* periodate buffered to pH 3.6, there was a rapid production of 0.4 mole of carbon dioxide.[34] A suggested explanation[33,34] of this result is the dissociation of the hydroxymalonaldehyde derivative to form mesoxalaldehyde (10) and an alcohol, the former then being rapidly oxidized to



(10)

carbon dioxide and formic acid. Whereas overoxidation proceeds to completion at pH 1 or 7.5, its rate at pH 3.5 may become extremely low. Cyclization of the hydroxyaldehyde intermediates is apparently responsible for this slowing down of the oxidation.[34]

Hough[33] has pointed out that some of the classical methods used for measuring periodate result in large changes in pH which can lead to erroneous results because of the hydrolysis of the intermediate esters. It is clear that the results obtained from periodate oxidation should always be interpreted with caution, attention being paid to the conditions of oxidation, particularly in unbuffered solutions.

Although overoxidation can lead to erroneous results, it can also be used for linkage analysis. Thus, Hough and Perry[31] showed that overoxidation, at pH 8, of dihexosaccharides containing a (1→2), (1→3), or (1→4) link yielded approximately 2 moles of formaldehyde per mole, but the corresponding disaccharides having other links yielded no formaldehyde. One mole of panose [*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose] gave 1 mole of formaldehyde, the (1→6) link blocking complete overoxidation. A similar technique was developed, in-

(33) L. Hough, T. J. Taylor, G. H. S. Thomas, and B. M. Woods, *J. Chem. Soc.*, 1212 (1958).

(34) M. Cantley, L. Hough, and A. O. Pittet, *Chem. & Ind.* (London), 1126, 1253 (1959).

TABLE II

Theoretical Yields of Carbon Dioxide and Formaldehyde Obtainable from Di- and Tri-(aldohexo)saccharides by Periodate Oxidation Under Controlled Conditions

Compound		*Reducing oligosaccharide*		*Reduced oligosaccharide*	
Glycosidic links	*Example*	CO_2 *(moles/mole at 50°, pH 5.0)*	*HCHO (moles/mole at 18–50°, pH 8.0)*	*HCHO (moles/mole)*	
				pH 3.6	*pH 8.0*
(1→1)	α,α-trehalose	—	—	0	0
(1→2)	sophorose	1	2	1	1
(1→3)	laminaribiose	1	2	2	3
(1→4)	maltose	1	2	2	3
(1→5)	—	0	0	1	1
(1→6)	isomaltose	0	0	1	1
(1→4)–(1→4)	cellotriose	2	3	2	4
(1→6)–(1→4)	panose	1	1	2	2

dependently, for examining the structures of dextrins.[35] Further structural information may be obtained by oxidizing the oligosaccharide alcohol (formed by borohydride reduction) with periodate at pH 8.0 and measuring the formaldehyde produced.[36] With this particular method, the alditol does not need to be isolated.

The carbon dioxide produced by stepwise overoxidation of reducing oligosaccharides with periodate (pH 5.0) at 50° has also been determined.[37] The method, although time-consuming, only requires 1 mg. of sugar. The rate of evolution of carbon dioxide may be increased by carrying out the oxidation at pH 1.0, but this acidity could cause hydrolysis of the oligosaccharides. The theoretical yields of carbon dioxide and formaldehyde resulting from overoxidation of a number of di- and tri-aldohexosaccharides are listed in Table II.

Overoxidation at pH 1.9 (at 50°) has also been used for measuring the D.P. of maltodextrins[38]; as measured by production of formaldehyde, equimolar amounts of the dextrins were oxidized at the same rate. Only 1–2 mg. of sugar is required and D.P.'s up to 100 can probably be measured.

(iii) *Miscellaneous Techniques.*—An examination of the fragments resulting from periodate oxidation (other than formic acid, formaldehyde,

(35) B. J. Bines and W. J. Whelan, *Biochem. J.*, **76**, 253 (1960).
(36) L. Hough, B. M. Woods, and M. B. Perry, *Chem. & Ind.* (London), 1100 (1957)
(37) L. Hough and B. M. Woods, *Chem. & Ind.* (London), 1421 (1957).
(38) F. W. Parrish and W. J. Whelan, *Nature*, **183**, 991 (1959).

and carbon dioxide) is helpful. In some oligosaccharides, the positions of glycosidic links may render individual monosaccharide residues stable to periodate oxidation, permitting them to be liberated intact after oxidation. Thus, the reducing, D-glucose residue of trisaccharide[9] (1) was unoxidized and recovered, by acid hydrolysis, after oxidation. A C-3-substituted D-glucopyranosyl residue in an oligosaccharide is also stable to periodate oxidation, and the D-glucose may be obtained by acid hydrolysis after oxidation. This method, combined with quantitative measurement of the liberated D-glucose, was used for demonstrating the presence of one (1→3) link, in the presence of two (1→6) links, in a D-glucose tetrasaccharide.[10] When periodate oxidation of a substituted monosaccharide residue is limited, hydrolysis of the oxidized residue may produce a lower monosaccharide. Thus, reduction of trisaccharide (1) to its alcohol, followed by periodate oxidation and hydrolysis, produces L-xylose from the original, reducing D-glucose residue.[9] In the case of α-D-glucopyranosyl α-D-galactofuranoside,[26] oxidation of one mole with only one mole of periodate, followed by borohydride reduction and acid hydrolysis, yielded D-glucose and L-arabinose, confirming the proposed structure and indicating that the D-galactosyl moiety of the original disaccharide is in the furanoside form.

The Barry degradation,[39] originally developed for studying polysaccharides, may be applied to oligosaccharides. By this method, the polyaldehyde resulting from periodate oxidation is treated with phenylhydrazine and acetic acid in order to split off the oxidized residues as phenylosazones. Monosaccharide residues stable to periodate are then recovered. The method has been used for investigating the structure of *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→2)-glycerol.[40]

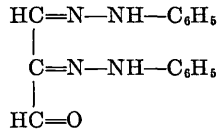
Treatment of periodate-oxidized oligosaccharides with bromine, to form the corresponding polycarboxylic acids, followed by hydrolysis and identification of the resulting acids of low molecular weight has also been used as a tool for structural studies.[20,27,41]

F. Smith and associates have used the polyhydric alcohols produced by periodate-oxidized, reducing carbohydrates in structural studies of polysaccharides; for a summary of this work, see Whelan.[42] With polysaccharides, two main procedures are used. First, examination of the alcohols of low molecular weight obtained by acid hydrolysis of the polyhydric alcohol; and second, methylation of the polyhydric alcohol, followed by hydrolysis and examination of the methylated alcohols. The molar ratios of

(39) V. C. Barry, *Nature*, **152**, 537 (1943).
(40) B. Lindberg, *Acta Chem. Scand.*, **9**, 1093 (1955).
(41) J. E. Courtois and A. Wickstrøm, *Bull. soc. chim. biol.*, **32**, 759 (1950).
(42) W. J. Whelan, *Ann. Rev. Biochem.*, **29**, 107 (1960).

glycerol to erythritol produced in the first method have also been used in order to give a measure of the ratio of intra-chain glycosyl residues to nonreducing terminal residues in polysaccharides.[43] So far, few oligosaccharides have been examined in this way,[44] but the method is likely to become important, particularly with saccharides of high molecular weight.

Periodate oxidation of oligosaccharide phenylosazones can also yield useful structural information. D-Glucose phenylosazone is thus oxidized to mesoxalaldehyde 1,2-bis(phenylhydrazone)[45] (11). The phenylosazones of



(11)

laminaribiose and turanose, having (1→3) links, and of maltose and cellobiose, having (1→4) links, do not produce aldehyde osazone (11), but it is precipitated from solution when (1→6)- or (1→5)-linked disaccharides are oxidized. These facts were used in establishing the structure of leucrose as 5-*O*-α-D-glucopyranosyl-D-fructopyranose.[46] The oxidation of other oligosaccharide osazones has been examined[47] and oligosaccharide phenylosatriazoles have also been used in similar, structural studies.[46] A quantitative micromethod for the oxidation of phenylosazones has been developed.[18]

Reducing oligosaccharides that are unsubstituted on C-2 or C-3 of the reducing glycose residue form 1-phenylflavazole derivatives, and the periodate oxidation of these derivatives may be used for indicating the presence of a (1→6) link[48] at the reducing end. In this case, oxidation of the phenylflavazole yields an insoluble 3-formyl-1-phenylflavazole; if, however, the link to the reducing end is (1→4), no aldehyde is obtained. The method was used to show that the link at the reducing end of manninotriose is (1→6).[48]

(43) J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956); I. J. Goldstein, J. K. Hamilton, R. Montgomery, and F. Smith, *ibid.*, **79**, 6469 (1957); M. Abdel-Akher and F. Smith, *Arch. Biochem. Biophys.*, **78**, 451 (1958).

(44) J. K. Hamilton, G. W. Huffman, and F. Smith, *J. Am. Chem. Soc.*, **81**, 2176 (1959); A. K. Mukherjee, D. Chaudhury, and P. Bagchi, *Can. J. Chem.*, **39**, 1408 (1961).

(45) E. Chargaff and B. Magasanik, *J. Am. Chem. Soc.*, **69**, 1459 (1947).

(46) F. H. Stodola, E. S. Sharpe, and H. J. Koepsell, *J. Am. Chem. Soc.*, **78**, 2514 (1956).

(47) G. Avigad, *Biochem. J.*, **73**, 587 (1959).

(48) D. French, G. M. Wild, and W. J. James, *J. Am. Chem. Soc.*, **75**, 3664 (1953).

OHLE et al. Ber. 74, 279 (1941).

c. Oxidation of Acidic and Basic Oligosaccharides.

(i) *Uronic Acid-containing Oligosaccharides.*—Periodate oxidation of these compounds has generally not been used, because of the difficulties introduced by overoxidation. These difficulties are, to some extent, overcome if the methyl esters are employed. Three procedures have been applied to aldobiouronic acids. First, oxidation of the methyl ester methyl glycoside[49]; second, conversion of this derivative, using lithium aluminum hydride[50] or potassium borohydride,[49] to the corresponding neutral disaccharides before oxidation; and, third, oxidation of the methyl glycoside of the aldobiouronamide.[51]

(ii) *Amino Sugar-containing Oligosaccharides.*—A comparatively small number of these oligosaccharides have been reported, and, consequently, not a great deal is known about their oxidation with periodate. In acid solution, oxidation of compounds containing a free amino group may be hindered because of protonation of the amino group.[52] The glycoside and alditol derivatives of the *N*-acetylated amino sugars undergo stoichiometric oxidation; for example, one mole of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside consumes one mole of oxidant, and one mole of 2-acetamido-2-deoxy-D-glucitol consumes 3 moles, with the production of 2 moles and 1 mole of formaldehyde and formic acid, respectively.[53] 2-Acetamido-2-deoxyhexoses can be overoxidized, probably by way of acetamidomalonaldehyde.[52] Thus *N*,*N*′-diacetylchitobiose [2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose] undergoes initial cleavage of the C-3 to C-4 glycol group of the nonreducing residue, followed by subsequent further oxidation at the reducing unit.[54] Amino sugar-containing oligosaccharide alditols containing a terminal 2-acetamido-2-deoxyalditol residue undergo normal oxidation without overoxidation,[53,54] and are the most reliable derivatives for structure determination. Borohydride reduction, followed by acid hydrolysis of the periodate-oxidized alditol yields a 3-, 4-, or 5-carbon 2-amino-2-deoxyalditol, according to the position of linkage to the alditol residue, and identification of this fragment affords a valuable direct confirmation of the linkage assign-

(49) G. O. Aspinall and I. M. Cairncross, *J. Chem. Soc.*, 3998 (1960).

(50) S. A. Barker, A. B. Foster, I. R. Siddiqui, and M. Stacey, *J. Chem. Soc.*, 2358 (1958).

(51) M. Abdel-Akher, F. Smith, and D. Spriestersbach, *J. Chem. Soc.*, 3637 (1952).

(52) M. Cantley and L. Hough, *Biochem. J.*, **77**, 6P (1960); G. E. McCasland and D. A. Smith, *J. Am. Chem. Soc.*, **73**, 5164 (1951).

(53) A. B. Foster and D. Horton, *J. Chem. Soc.*, 1890 (1958).

(54) S. A. Barker, A. B. Foster, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 2218 (1958).

ment based on the oxidation data.[53,55] When the terminal residue is not an amino sugar, overoxidation of the derived alditol may occur; thus 3-*O*-(2-acetamido-2-deoxy-β-D-glucosyl)-D-galactitol, which initially gives a malonaldehyde derivative from the alditol residue, undergoes overoxidation.[55a]

4. *Lead Tetraacetate and Other Oxidation Methods*

Lead tetraacetate performs the same oxidative cleavage of 1,2-glycol groups as periodate, and its action is closely related to that of periodate. The oxidation of carbohydrates by lead tetraacetate has been reviewed in considerable detail by Perlin,[56] and only brief mention of it need be made here. As with periodate oxidation, much useful structural information about an oligosaccharide is obtained by measuring tetraacetate consumption, production of formic acid and formaldehyde, and oxidation rates. In addition, under carefully controlled conditions, tetraacetate oxidation has been used for determining the α or β configuration of the glycosidic links in (1→2)-, (1→3)-, and (1→4)-linked disaccharides. In this method, the sugars are converted to glycerol glycosides which can be compared with authentic specimens.[56] This useful degradation has also been extended to higher oligosaccharides.[56]

Cuprimetric reagents[57] are normally too unspecific in their action to be of much value for investigating oligosaccharide structures. However, if the link on the reducing monosaccharide residue of an oligosaccharide is (1→2), the sugar is virtually nonreducing to the Shaffer–Hartmann copper reagent,[57] although carbon atom 1 can be oxidized by the theoretical amount of such reagents as alkaline hypoiodite.[9] These two oxidants can, therefore, provide a useful indication of the presence of a (1→2) link at the reducing end of a molecule.[9] Comparison of the copper-reducing value of an oligosaccharide with that of its reducing monosaccharide unit can give an indication of D.P., provided that the link on the reducing unit is (1→4), (1→5), or (1→6) and that the correct heating time is used with the appropriate copper reagent.[58,59] Alkaline hypoiodite oxidizes only the reducing carbon atom of a sugar; hence, its action is unaffected by the

(55) A. B. Foster, D. Horton, N. Salim, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 2587 (1960).

(55a) S. A. Barker, M. Heidelberger, M. Stacey, and D. J. Tipper, *J. Chem. Soc.*, 3468 (1958).

(56) A. S. Perlin, *Advances in Carbohydrate Chem.*, **14**, 9 (1959).

(57) P. A. Shaffer and A. F. Hartmann, *J. Biol. Chem.*, **124**, 425 (1921); M. Somogyi, *ibid.*, **160**, 61 (1945).

(58) J. R. Turvey and W. J. Whelan, *Biochem. J.*, **67**, 49 (1957).

(59) R. W. Bailey and R. T. J. Clarke, *Biochem. J.*, **72**, 49 (1959).

glycosidic link on the reducing monosaccharide residue of an oligosaccharide, and it can, therefore, be used[9] to measure D.P.

5. *Methylation Analysis*

Methylation continues to be a standard method for structural investigations. In brief, complete methylation of an oligosaccharide converts all unsubstituted hydroxyl groups to methoxyl groups, and each free hydroxyl group present in the methylated monosaccharides that are liberated by hydrolysis corresponds to a substituted hydroxyl group of the original oligosaccharide. The method, therefore, defines (not necessarily unambiguously) the position of the glycosidic links and the ring sizes of the individual glycose residues.

The ideal methylating agent is one which can rapidly introduce the theoretical number of methyl groups and, at the same time, cause no degradation of the sugar. Unfortunately, it is difficult to find reagents with these properties and, generally, several methylations with two different reagents are necessary to obtain full methylation. Several classical techniques are available for methylation. These include refluxing with methyl iodide and silver oxide[60] (Purdie's method), treatment with dimethyl sulfate and aqueous sodium hydroxide[61] (Haworth's method), conversion of the sugar to a thallium derivative followed by treatment with methyl iodide,[62] and reaction with an alkali metal and methyl iodide in liquid ammonia.[63]

Haworth's method is probably the most widely used in the oligosaccharide field, and is commonly employed in conjunction with Purdie's method. Partially methylated sucrose obtained by the Haworth procedure has also been fully methylated by forming the sodium derivative in ether followed by reaction with methyl iodide,[64] and the liquid ammonia procedure has been used in conjunction with Haworth's method in the case of raffinose hendecaacetate.[65] Diazomethane can methylate hydroxyl groups under very mild conditions, but, even with simple compounds, the reaction is incomplete.[66] It has, however, been used for methylating the carboxyl group of an aldobiouronic acid following methylation of the hydroxyl groups with dimethyl sulfate.[67] The main disadvantage of all of these

(60) T. Purdie and J. C. Irvine, *J. Chem. Soc.*, **83,** 1021 (1903).
(61) W. N. Haworth, *J. Chem. Soc.*, **107,** 8 (1915).
(62) C. M. Fear and R. C. Menzies, *J. Chem. Soc.*, 937 (1926).
(63) I. E. Muskat, *J. Am. Chem. Soc.*, **56,** 695 (1934).
(64) E. Pacsu and S. M. Trister, *J. Am. Chem. Soc.*, **61,** 2442 (1939).
(65) K. Hess and K.-H. Lung, *Ber.*, **71,** 827 (1938).
(66) L. Hough and J. K. N. Jones, *Chem. & Ind.* (London), 380 (1952).
(67) P. A. Levene, G. M. Meyer, and M. Kuna, *J. Biol. Chem.*, **125,** 703 (1938).

methylation methods is that they are not suitable for microscale work with 1–2 mg. of sugar.

An important contribution to methylation technique has been made by Kuhn and associates,[68] who have methylated sucrose in high yield by using methyl iodide and silver oxide in *N*,*N*-dimethylformamide. The temperature is not allowed to rise above 30° and reducing oligosaccharides appear to be unaffected by the silver oxide. Perila and Bishop,[69] using this procedure with cellobiose, could find no acidic products which might have resulted from oxidation with silver oxide; in Purdie's method, oxidation of reducing sugars can occur.[70] Recently, in the methylation of acetamido sugars, silver oxide has been replaced by barium oxide, as the latter does not degrade *N*-acetyl groups.[71] Many oligosaccharides are soluble in *N*,*N*-dimethylformamide, so this methylation procedure should prove useful, although, up to now, it has not been widely applied.

Column and paper-chromatographic methods for the separation, identification, and estimation of methylated monosaccharides have been developed,[2] and are satisfactory, provided that the appropriate reference compounds are available. Recent developments in this field are the ready demethylation of methylated sugars with boron trichloride[72,73] and the use of gas–liquid partition chromatography for the analysis of mixtures of methylated sugars.[74]

Some idea of the future trend of methylation analysis can, perhaps, be gained from the work of Perila and Bishop,[69] who have used the method to study oligosaccharides obtained by the enzymic hydrolysis of jack-pine mannan. The oligosaccharides (0.5–2.0 mg.) were methylated by the Kuhn procedure,[68] refluxed with methanolic hydrogen chloride, and the resulting methyl *O*-methylglycosides analyzed qualitatively and quantitatively by gas–liquid chromatography.

6. *Enzymic Methods*

The widespread use of enzymes in Chemistry is apparent on inspection of a recently published list[75] entitled "Enzymes Commonly Used as Lab-

(68) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).

(69) O. Perila and C. T. Bishop, *Can. J. Chem.*, **39**, 815 (1961).

(70) W. N. Haworth and G. C. Leitch, *J. Chem. Soc.*, **113**, 188 (1918).

(71) R. Kuhn, H. H. Baer, and A. Seeliger, *Ann.*, **611**, 236 (1958).

(72) S. Allen, T. G. Bonner, E. J. Bourne, and N. M. Saville, *Chem. & Ind.* (London), 630 (1958).

(73) T. G. Bonner, E. J. Bourne, and S. McNally, *J. Chem. Soc.*, 2929 (1960).

(74) A. G. McInnes, D. H. Ball, F. R. Cooper, and C. T. Bishop, *J. Chromatog.*, **1**, 556 (1958); C. T. Bishop and F. P. Cooper, *Can. J. Chem.*, **38**, 388, 793 (1960); C. T. Bishop, F. Blank, and P. E. Gardner, *ibid.*, **38**, 869 (1960).

(75) W. H. Elliot, in "Data for Biochemical Research," R. M. C. Dawson, D. C. Elliot, W. H. Elliot, and K. M. Jones, eds., Oxford University Press, Oxford, Engl., 1959, p. 178.

oratory Reagents." With oligosaccharides, the hydrolases are the most important group of enzymes used in structural and preparative work. Future development of enzymic techniques would most certainly be assisted by adequate commercial sources of many of the enzymes; most of them, particularly in highly purified forms, are neither cheap nor easy to obtain.

The particular value of enzymic methods is the high degree of specificity often obtainable as regards hydrolysis of the anomeric configuration of the glycosidic link. Also, the reactions proceed rapidly at low temperatures (25–35°) and at pH values not far removed from neutrality. There is evidence that at least some hydrolases also function in the presence of relatively high concentrations of polar organic solvents.[76] Used in conjunction with paper chromatography, small-scale enzymic digests can provide useful information from very small amounts of sugar. The digest may be examined, by paper chromatography, during the reaction; or, on completion of the reaction, enzyme protein may normally be readily removed and the products isolated. The main dangers which arise in interpreting the results of enzymic studies can be avoided by a careful characterization of the enzyme. In particular, its specificity should always be checked with control digests of a suitable range of disaccharides. The possibility of oligosaccharide synthesis by transglycosylation should also be checked; this latter possibility is generally obviated by the use of a very low concentration of sugar.

Few, if any, simple glycosidases have been crystallized, and, indeed, for many hydrolyses, extremely complex mixtures of enzymes, for example, almond emulsin, are often used. The specificity of such complex mixtures may be increased not only by fractionation, but by the addition of appropriate inhibitors. Thus, commercial hemicellulase normally hydrolyzes both the L-arabinose–D-xylose and the D-xylose–D-xylose link in arabinoxylans. Addition of L-arabinono-1,4-lactone to the enzyme digest, however, inhibits hydrolysis of the former links (see Conchie and Levvy[77]) and *O*-L-arabinofuranosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose can be obtained.[78] The lability of the furanoid residue does not allow oligosaccharides containing such residues to be obtained by partial acid hydrolysis. This enzymic method should prove of value in structural studies on oligosaccharides; thus, the hydrolysis of α-D-glucopyranosyl α-D-galactofuranoside by a mixture of α-glucosidase and α-galactosidase was shown to be due to the α-glucosidase, because, on inhibition of this enzyme with D-

(76) E. T. Reese and M. Mandels, *J. Am. Chem. Soc.*, **80**, 4625 (1958); A. I. Tiffen, unpublished results.

(77) J. Conchie and G. A. Levvy, *Biochem. J.*, **65**, 389 (1957).

(78) G. O. Aspinall, I. M. Cairncross, R. J. Sturgeon, and K. C. B. Wilkie, *J. Chem. Soc.*, 3881 (1960).

glucurono-6,3-lactone, there was no hydrolysis.[26] With all enzyme studies of this type, it would be advisable to include control digests containing specific inhibitors, if available, as a further check on the specificity of hydrolysis.

A detailed description of all the carbohydrases used in oligosaccharide studies is not possible in the present review; further information concerning sources, specificities, and properties of these enzymes may be found elsewhere.[79] Examples of the use of the two general classes of hydrolases, glycosidases, and polysaccharidases are given below.

a. Glycosidases.—Glycosidases (α- and β-D-), from both plant and animal sources, that have various degrees of hydrolytic specificity with regard to the position of glycosidic links, are commonly used to provide evidence concerning the configuration of these links. Thus, almond emulsin, which will not hydrolyze α-D-glucosides, has been used to show the presence or absence of β-D-links in many D-glucose-containing oligosaccharides; for example, di- and tri-saccharides,[26,80] a partially methylated di-D-glucosaccharide,[81] an *O*-D-glucosyl-D-xylose,[82] and a disaccharide glycoside.[83] Failure of emulsin to hydrolyze an oligosaccharide does not necessarily mean that β-D-links are absent. The β-D-glucosidase liberates D-glucose from the substrate in a stepwise manner, from the nonreducing end; thus, 3-*O*-β-isomaltosyl-D-glucose is unattacked because of the α-D-link at the nonreducing end.[84] Shielding of the β-D-link can also occur, as with the branched trisaccharide (1), which is only slowly hydrolyzed by β-D-glucosidase.[9] Reduction of (1) to the alditol, however, permits hydrolysis to proceed at a normal rate.[9] Substitution of the hydroxyl groups of a β-D-glucopyranosyl residue affects the rate of hydrolysis, inhibition being particularly marked[78] with substituents on C-2 and C-3. Almond β-D-glucosidase is also used for partial-hydrolysis studies. The isomeric trisaccharides 3-*O*-β-cellobiosyl-D-glucose and 4-*O*-β-laminaribiosyl-D-glucose were readily identified by this means. When treated with the enzyme,

(79) C. Neuberg, I. Mandel, A. Gottschalk, S. Viebel, and W. Fishman, in "The Enzymes; Chemistry and Mechanism of Action," J. B. Sumner and K. Myrbäck, eds., Academic Press Inc., New York, N. Y., 1950, Vol. 1, Pt. 1; A. Gottschalk, in "Encyclopedia of Plant Physiology," W. Ruhland, ed., Springer-Verlag, Berlin, Ger., 1958, Vol. 6, p. 87; W. J. Whelan, *ibid.*, p. 190; "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrbäck, eds., Academic Press Inc., New York, N. Y., 1960, Vol. 4.

(80) F. B. Anderson and D. J. Manners, *Biochem. J.*, **71**, 407 (1959).

(81) S. A. Barker, E. J. Bourne, P. M. Grant, and M. Stacey, *J. Chem. Soc.*, 601 (1958).

(82) W. A. M. Duncan, D. J. Manners, and J. L. Thompson, *Biochem. J.*, **73**, 295 (1959).

(83) J. D. Anderson, L. Hough, and J. B. Pridham, *Biochem. J.*, **77**, 564 (1960).

(84) S. Peat, W. J. Whelan, and H. G. Lawley, *J. Chem. Soc.*, 724, 729 (1958).

both gave D-glucose, but each gave a different disaccharide—laminaribiose and cellobiose, respectively.[85] 3-*O*-β-Gentiobiosyl-D-glucose can also be identified by partial hydrolysis with acid and emulsin; whereas treatment with acid yields D-glucose, laminaribiose, and gentiobiose, the specific action of emulsin produces only one disaccharide, laminaribiose.[84]

Almond emulsin also contains an α-D-galactosidase component[85a] which has been used for structural studies with stachyose[48,86] and planteose.[87] Both oligosaccharides give D-galactose and the expected, partial-hydrolysis products resulting from the stepwise cleavage of α-D-galactopyranosyl links. A better source of α-D-galactosidase is the coffee bean. This preparation, like almond emulsin, possesses little or no β-D-fructofuranosidase activity and only a very weak β-D-galactosidase activity. It has been used extensively by Courtois and his coworkers in studies on naturally occurring *O*-D-galactosylsucrose derivatives.[88]

A third enzyme useful for oligosaccharide hydrolysis is α-D-glucosidase, the chief source of which is yeast.[78] This enzyme has also been used for checking the configuration of anomeric carbon atoms.[26,89] Avigad[47] has prepared β-D-fructofuranosidase-free enzyme from dried yeast-cells and used it to detect α-D-links in a number of mono- and di-*O*-D-glucosylfructose oligosaccharides. Intestinal α-D-glucosidase,[87] and honey invertase[90] are also useful agents for the hydrolysis of α-D-links.

Yeast is also a source of invertase, the much-used β-D-fructofuranosidase which hydrolyzes sucrose and which is specific only for the D-fructose moiety of this disaccharide. Sucrose derivatives having D-fructofuranoside end-groups are also hydrolyzed by the enzyme. In conjunction with paper chromatography, hydrolysis with invertase is a useful aid to the identification of small amounts of these sugars.[91,92] With increasing D.P. of *O*-D-galactosylsucrose derivatives, the rate of hydrolysis of the D-fructose residue decreases; thus, raffinose is hydrolyzed faster than stachyose.[88]

(85) S. Peat, W. J. Whelan, and J. G. Roberts, *J. Chem. Soc.*, 3916 (1957).

(85a) K. Wallenfels and O. P. Malhotra, *Advances in Carbohydrate Chem.*, **16**, 239 (1961).

(86) C. Neuberg, *Biochem. Z.*, **3**, 528 (1910).

(87) D. French, G. M. Wild, B. Young, and W. J. James, *J. Am. Chem. Soc.*, **75**, 709 (1953).

(88) J. E. Courtois, *Proc. Intern. Congr. Biochem. 4th Congr. Vienna*, 1958, **1**, 147 (1959).

(89) M. Bridel and T. Aagaard, *Bull. soc. chim. biol.*, **9**, 884 (1927).

(90) J. M. Nelson and D. J. Cohn, *J. Biol. Chem.*, **61**, 193 (1924); J. B. Pridham, *Chem. & Ind.* (London), 1172 (1961).

(91) R. W. Henderson, R. K. Morton, and W. A. Rawlinson, *Biochem. J.*, **72**, 340 (1959); S. Haq and G. A. Adams, *Can. J. Chem.*, **39**, 1165 (1961).

(92) J. B. Pridham, *Biochem. J.*, **76**, 13 (1960).

When the D-fructose unit of sucrose is substituted, as in planteose,[87,93] lychnose,[88] or melezitose,[94] hydrolysis does not occur.

The foregoing discussion has been principally concerned with the use of glycosidases to demonstrate the anomeric configuration of glycosidic links. Many enzymes are specific for a particular glycosidic link (for example, maltase, cellobiase) and, when available, can be used to identify these sugars. Several glycosidases seem to have been largely ignored by carbohydrate chemists. β-D-Glucuronidase,[94a] for example, which occurs widely in animal tissues[78] could possibly be a useful hydrolyst for aldobiouronic acids. Several so-called disaccharidases have been reported which hydrolyze a disaccharide glycoside to disaccharide and aglycon,[95–97] possibly because of different configurations of the two glycosidic carbon atoms. These enzymes seem worth investigating further.

b. Polysaccharidases.—Enzymes associated with degradation of polysaccharides can generally hydrolyze related oligosaccharides; their use with oligosaccharides is illustrated here by reference to the *amylolytic enzymes.* α-Amylase, which randomly cleaves the α-D-(1→4) links of starch polymers, hydrolyzes maltodextrins of D.P. 4 (or higher) to mixtures of maltose and maltotriose. As the ratio of these two products is determined by the D.P. of the maltodextrin, α-amylase action can be used to determine the D.P. of a maltodextrin.[98] β-Amylase, which cleaves alternate α-D-(1→4) links (from the nonreducing end of the molecule) to liberate maltose, can also be used to check the D.P. of a maltodextrin.[99] Glucamylase (amyloglucosidase) catalyzes the cleavage of single D-glucose residues from the nonreducing end of the starch molecule.[100] The presence of an α-D-(1→3)-, α-D-(1→6)-, or β-D-link in an α-D-(1→4)-linked oligo-D-glucoside stops its action; thus, whereas *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose is not attacked by glucamylase, *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucose is hydrolyzed to nigerose and D-glucose.[100] R-Enzyme hydrolyzes many of

(93) N. Wattiez and M. Hans, *Bull. acad. roy. méd. Belg.*, **8**, 386 (1943).

(94) M. Adams, N. K. Richtmyer, and C. S. Hudson, *J. Am. Chem. Soc.*, **65**, 1369 (1943).

(94a) G. A. Levvy and C. A. Marsh, *Advances in Carbohydrate Chem.*, **14**, 381 (1959).

(95) C. Charaux, *Compt. rend.*, **178**, 1312 (1924); M. Bridel, *ibid.*, **179**, 780 (1924).

(96) H. Imaseki and T. Yamamoto, *Arch. Biochem. Biophys.*, **92**, 467 (1961).

(97) D. W. S. Westlake, G. Talbot, E. R. Blakely, and F. J. Simpson, *Can. J. Microbiol.*, **5**, 621 (1959).

(98) W. J. Whelan and P. J. P. Roberts, *J. Chem. Soc.*, 1293, 1298 (1953).

(99) W. J. Whelan and J. G. Roberts, *Biochem. J.*, **58**, 569 (1954).

(100) S. A. Barker and J. G. Fleetwood, *J. Chem. Soc.*, 4857 (1957); S. A. Barker, E. J. Bourne, and J. G. Fleetwood, *ibid.*, 4865 (1957). See D. J. Manners, *Advances in Carbohydrate Chem.*, **17**, 371 (1962).

the α-D-(1→6) branch links of starch polysaccharides, its action being specific for an α-D-(1→6) link present with a certain association of α-D-(1→4) links. It has been used in investigating a pentasaccharidic limit-dextrin containing one α-D-(1→6) and three α-D-(1→4) links.[35]

As with the amylases, such other enzymes as dextranases,[59] cellulases,[79] and hemicellulases[79] can be used to study oligosaccharides whose structure is closely related to the relevant polysaccharide. The particular value of these enzymes is that their specificity depends on the position of substitution of the glycosidic links as well as on their configuration.

7. *Miscellaneous Methods*

Measurement of optical activity enables the configuration of a glycosidic link to be deduced from the specific optical activity of the oligosaccharide[30]; or, by the use of Hudson's isorotation rules,[101] by comparison with the specific optical rotation[102] of known isomers, and by the change in optical activity[103,104] on hydrolysis. The marked effect of borate on the optical rotation of di- and tri-saccharides[105] may also be of value in ascertaining the configuration or position of the glycosidic link. In a homologous series of oligosaccharides, such properties as specific optical rotation,[58,106] reducing power,[58] and movement on paper chromatograms[107] show a linear relationship with D.P., a relationship which may be used to test whether a compound is a member of such a series.

Infrared spectra have been mainly used in structural studies on polysaccharides; they are, however, of some value in similar studies on oligosaccharides, particularly if D-glucopyranosyl residues are present. The infrared spectra of a number of D-glucosyl oligosaccharides, over the frequency range of 730–960 cm.$^{-1}$ have been reported,[108] and Barker, Bourne, and Theander[102] were able to show that the infrared spectrum of *O*-α-D-glucopyranosyl-(1→6)-α-D-glycopyranosyl β-D-fructofuranoside is almost a summation of the spectra of isomaltose and sucrose. Infrared spectra of D-glucose-containing oligosaccharides also exhibit absorption bands at 840 ± 8 cm.$^{-1}$ and 894 ± 7 cm.$^{-1}$ which are said to be characteristic of α- and β-D-links, respectively. The presence or absence of these bands

(101) C. S. Hudson, *J. Am. Chem. Soc.*, **31,** 66 (1909); **38,** 1566 (1916).
(102) S. A. Barker, E. J. Bourne, and O. Theander, *J. Chem. Soc.*, 2064 (1957).
(103) S. A. Barker, M. Stacey, and D. B. E. Stroud, *Nature*, **189,** 138 (1961).
(104) E. W. Putman, C. Fitting-Litt, and W. Z. Hassid, *J. Am. Chem. Soc.*, **77,** 4351 (1955).
(105) R. C. Hughes and W. J. Whelan, *Chem. & Ind.* (London), 50 (1959).
(106) M. L. Wolfrom and J. C. Dacons, *J. Am. Chem. Soc.*, **74,** 5331 (1952).
(107) D. French and G. M. Wild, *J. Am. Chem. Soc.*, **75,** 2612 (1953).
(108) S. A. Barker, E. J. Bourne, M. Stacey, and D. H. Whiffen, *J. Chem. Soc.*, 171 (1954).

has been used to confirm the presence of α- and β-D-links in certain trisaccharides[9,102] and of α-D-links in D-glucose-,[109] and *O*-D-glucosyl-D-xylose disaccharides.[103] This procedure is of most value when used with disaccharide alditols, since otherwise the absorption bands associated with the reducing group will be superimposed on those associated with the configuration at C-1 of the nonreducing group.[53,54]

The effect of alkali on oligosaccharides has been reviewed by Whistler and BeMiller.[110] In addition to epimerization reactions, alkali degrades some oligosaccharides in a stepwise manner from the reducing end of the molecule. Oligosaccharides which have substituted hydroxyl groups on C-3, C-4, or C-6 of the reducing end-group are degraded to metasaccharinic, isosaccharinic, and (mainly) lactic acids, respectively, whereas similar substitution on C-2 inhibits degradation. The procedure has not been used widely as an analytical method, but it can be used to detect (1→2) links and for a stepwise "peeling off" of the monosaccharide residues. Thus *O*-L-galactopyranosyl-(1→4)-*O*-D-xylopyranosyl-(1→2)-L-arabinose is stable[111] to lime-water at 25°, and alkaline degradation of *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose yields 2-*O*-α-L-fucopyranosyl-D-galactose.[112] Stepwise degradation of xylotriose[113] and cellotetraose[114] by lime-water has been described.

The catalytic oxidation of primary hydroxyl groups to carboxyl groups has been used for stabilizing acid-labile links in polysaccharides.[49] In this way, an L-arabinofuranosyl residue is converted to an L-arabinofuranosyluronic acid residue, and an acid-stable aldobiouronic acid may be isolated. This technique might be applicable to oligosaccharides having acid-labile links. The reduction of a methyl ester methyl glycoside to a neutral disaccharide prior to periodate oxidation[49,50] can be used for converting a uronic acid oligosaccharide to a neutral oligosaccharide[115] which may be a known compound,[115] or which can be used in partial, acid-hydrolysis studies. A method for the measurement of uronic acid residues in uronic acid oligosaccharides has been described by Barker, Foster, Siddiqui, and Stacey.[116]

Oligosaccharides, particularly in the D-glucose series, may often be

(109) S. Haq and W. J. Whelan, *Nature*, **178**, 1222 (1956).

(110) R. L. Whistler and J. N. BeMiller, *Advances in Carbohydrate Chem.*, **13**, 289 (1958).

(111) R. L. Whistler and W. M. Corbett, *J. Am. Chem. Soc.*, **77**, 6328 (1955).

(112) R. Kuhn, H. H. Baer, and A. Gauhe, *Ann.*, **611**, 242 (1958).

(113) R. L. Whistler and W. M. Corbett, *J. Am. Chem. Soc.*, **78**, 1003 (1956).

(114) W. M. Corbett and J. Kenner, *J. Chem. Soc.*, 1431 (1955).

(115) S. A. Barker, A. Gómez-Sánchez, and M. Stacey, *J. Chem. Soc.*, 3264 (1959).

(116) S. A. Barker, A. B. Foster, I. A. Siddiqui, and M. Stacey, *Talanta*, **1**, 216 (1958).

readily identified as their acetates. Wolfrom and his colleagues have developed methods for separating mixtures of these compounds and have used the procedure for identifying partial-hydrolysis products from polysaccharides.[106,117]

Acetates of oligosaccharides composed of (1→6)-linked D-glucopyranosyl residues give a characteristic change in optical rotation when treated with hydrogen bromide in acetic acid and acetyl bromide.[118] The conversion of two disaccharides to the same osazone means that the glycosidic links are the same; thus, nigerose and turanose give the same osazone.[119] Production of a monosaccharide osazone from an oligosaccharide indicates a (1→2) link at the reducing end of the molecule,[9] although other links [for example, (1→4)] can be hydrolyzed to some extent[120] by this means. Aldohexopyranosyl oligosaccharides having a (1→4) link at the reducing end of the molecule cannot form methyl furanosides when treated with methanolic hydrochloric acid, whereas (1→3)-linked compounds of this type can. This fact has been used for the separation of two closely related trisaccharides[121] and for showing that an *O*-D-glucosyl-D-xylose disaccharide was (1→3)- and not (1→4)-linked.[122]

The Morgan–Elson reaction for the determination[123] of 2-acetamido sugars can be used for obtaining an indication of the position of the glycosidic links in 2-acetamido-2-deoxy-D-glucose oligosaccharides.[124] It has been used in this way to show the presence of (1→6) links[53,125] and (1→4) links[54,125a] Hyalobiouronic acid was ultimately converted to a neutral *O*-D-glucosyl-L-arabinose disaccharide by oxidative degradation with ninhydrin,[126] and this technique may be of value with other amino sugar oligosaccharides. The studies of Kabat and others[127] suggest that, in the

(117) M. L. Wolfrom and A. Thompson, *J. Am. Chem. Soc.*, **79**, 4212 (1957); **80**, 6618 (1958).

(118) A. Jeanes, C. A. Wilham, R. W. Jones, H. M. Tsuchiya, and C. E. Rist, *J. Am. Chem. Soc.*, **75**, 5911 (1953).

(119) S. Haq and W. J. Whelan, *J. Chem. Soc.*, 1342 (1958).

(120) E. J. Bourne, J. Hartigan, and H. Weigel, *J. Chem. Soc.*, 2332 (1959).

(121) S. A. Barker, E. J. Bourne, D. M. O'Mant, and M. Stacey, *J. Chem. Soc.*, 2448 (1957).

(122) S. A. Barker, E. J. Bourne, G. C. Hewitt, and M. Stacey, *J. Chem. Soc.*, 3541 (1957).

(123) W. T. J. Morgan and L. A. Elson, *Biochem. J.*, **28**, 988 (1934).

(124) R. W. Jeanloz and M. Trémège, *Federation Proc.*, **15**, 282 (1956); see also, Ref. 128.

(125) I. Danishefsky, H. B. Eiber, and E. Langholtz, *Biochem. Biophys. Research Communs.*, **3**, 571 (1960).

(125a) M. L. Wolfrom, J. R. Vercellotti, and D. Horton, *J. Org. Chem.*, **27**, 705 (1962).

(126) B. Weissmann and K. Meyer, *J. Am. Chem. Soc.*, **76**, 1753 (1954).

(127) P. Z. Allen and E. A. Kabat, *J. Am. Chem. Soc.*, **81**, 4382 (1959), and other literature cited therein.

not-too-distant future, it may be possible to identify some oligosaccharides by simple precipitin reactions with antisera.

Examples of methods cited in the present article have been chosen from a limited number of publications; many more examples may be found in the post-1945 References listed in Tables III–V. Chemical synthesis of oligosaccharides[1] may often be used to give a final proof of structure as, for example, with nigerose.[119] Prediction of structure from the known transferring properties of an enzyme should be used with caution; thus, dextransucrase preparations, which normally transfer D-glucose [to form α-D-(1→6) links], have now been shown to transfer D-glucose to C-2 of a β-D-linked D-glucopyranosyl residue,[9] C-5 of D-fructopyranose,[46] and C-1 of D-galactofuranose.[26]

III. Tables of Oligosaccharides

1. *Compounds Included*

The Tables list oligosaccharide polymers of simple monosaccharides and of the following naturally occurring derivatives: amino sugars, 2-acetamido sugars, uronic acids, and mono-*O*-methyluronic acids. Although it was considered desirable to include only compounds whose structures had been established beyond doubt, such a rule would have excluded many important compounds. Exceptions to this rule were, therefore, made for compounds of incompletely established structure, provided that there was sufficient evidence to show that they were unlikely to be compounds already known.

2. *Classification*

The oligosaccharides are classified in three Tables according to the following scheme.

Table III. *Oligosaccharides Containing Simple Monosaccharides Only*

a. *Homogeneous* with respect to the glycosyl units
b. *Heterogeneous* with respect to the glycosyl units
   (1) Reducing
   (2) Nonreducing

Table IV. *Oligosaccharides Containing an Amino Sugar*

a. Containing an amino sugar and simple monosaccharides
b. Containing an amino sugar and uronic acids

Table V. *Oligosaccharides Containing a Uronic Acid*

a. Containing a uronic acid and simple monosaccharides
b. Naturally occurring mono-*O*-methyl derivatives of these oligosaccharides

Where possible, compounds are listed in alphabetical order, starting from the nonreducing end of the molecule. Compounds in Table III,b,(2), and, to some extent, in Tables IV and V, are, however, divided into structural groups. Within each isomeric series, the sugars are listed in numerical order with respect to the glycosidic links. For each homologous series, two entries are made where justified—one for the initial member and one for all the higher members. The latter are indicated by the following bracketing scheme: *O*-α-D-G*p*(1→[4-*O*-α-D-G*p*-1]$_n$→4)-D-G. Branched oligosaccharides are indicated by the bracketing scheme: *O*-α-D-G*p*(1→4)-*O*-[-α-D-G*p*-(1→3)]-D-G.

3. *Source*

The source is recorded according to the following code: A, occurring free in Nature; B, obtained from the chemical or enzymic hydrolyzate of a higher polymer; C, synthesized chemically (including acid reversion); D, synthesized enzymically *in vitro* (including bacterial cultures).

One reference is given, if known, for each type of source recorded. Where many references are available, a reference has been selected which either gives the full details of the sugar or reviews all of the earlier literature. References not given in the preceding text are listed at the end of the Tables.

TABLE III

Oligosaccharides Composed of Simple Monosaccharides

a. Homogenous Compounds

Oligosaccharide	*Source*	*References*
D-Arabinose		
α-D-Arabinopyranosyl α-D-arabinopyranoside	C	129
L-Arabinose		
3-*O*-L-Arabinofuranosyl-L-arabinose	B	130
5-*O*-L-Arabinofuranosyl-L-arabinofuranose	B	130
β-L-Arabinopyranosyl β-L-arabinopyranoside	C	131
3-*O*-β-L-Arabinopyranosyl-L-arabinose	B, C	131, 132
4-*O*-β-L-Arabinopyranosyl-L-arabinose	C	131
5-*O*-α-L-Arabinopyranosyl-L-arabinose	B	133
D-Fructose		
1-*O*-β-D-Fructofuranosyl-D-fructose (Inulobiose)	A, B, D	134–136
O-β-D-Fru*f*-(2→[1-*O*-β-D-Fru*f*-2]$_n$→1)-D-Fru, n = 2–4 (Inulodextrins)	B	135

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
6-*O*-β-D-Fructofuranosyl-D-fructose (Levanbiose)	B, D	136, 137
O-β-D-Fru*f*-(2→[6-*O*-β-D-Fru*f*-2]$_n$→6)-D-Fru, n = 1–8	B	138
O-β-D-Fru*f*-(2→[6-*O*-β-D-Fru*f*-2]$_n$→6)-*O*-β-D-Fru*f*-(2→1)-D-Fru, n = 1–8	B	138
L-Fucose (6-deoxy-L-galactose)		
2-*O*-α-L-Fucopyranosyl-L-fucose	B	139
3-*O*-α-L-Fucopyranosyl-L-fucose	B	139
4-*O*-α-L-Fucopyranosyl-L-fucose	B	139
D-Galactose		
5-*O*-α-D-Galactosyl-D-galactose	D	140
5-*O*-β-D-Galactofuranosyl-D-galactose	B	141
O-β-D-Gal*f*(1→[5-*O*-β-D-Gal*f*-1]$_n$→5)-D-Gal, n = 1–3	B	141
α-D-Galactopyranosyl α-D-galactopyranoside	C	142
α-D-Galactopyranosyl β-D-galactopyranoside	C	143
β-D-Galactopyranosyl β-D-galactopyranoside	C	142
3-*O*-α-D-Galactopyranosyl-D-galactose	B, D	140, 144
O-α-D-Gal*p*-(1→3)-*O*-α-D-Gal*p*-(1→3)-D-Gal	B	144
3-*O*-β-D-Galactopyranosyl-D-galactose	B, C, D	145–147
O-β-D-Gal*p*-(1→3)-*O*-β-D-Gal*p*-(1→3)-D-Gal	B	145
O-β-D-Gal*p*-(1→3)-*O*-β-D-Gal*p*-(1→6)-D-Gal	B	145
4-*O*-α-D-Galactopyranosyl-D-galactopyranose	B, D	140, 148
4-*O*-β-D-Galactopyranosyl-D-galactose	B, C	149, 150
O-β-D-Gal*p*(1→[4-*O*-β-DGal*p*-1]$_n$→4)-D-Gal, n = 1–7	B	151
6-*O*-α-D-Galactopyranosyl-D-galactopyranose (Galactobiose)	A, B, C, D	107, 140, 152, 153
O-α-D-Gal*p*-(1→6)-*O*-α-D-Gal*p*-(1→6)-D-Gal*p*	C	107
6-*O*-β-D-Galactopyranosyl-D-galactopyranose	B, C, D	154–156
O-β-D-Gal*p*-(1→[6-*O*-β-D-Gal*p*-1]$_n$→6)-D-Gal*p*, n = 1 or 2	B	154
D-Glucose		
α-D-Glucopyranosyl α-D-glucopyranoside (α,α-Trehalose)	A, C, D	157–159
α-D-Glucopyranosyl β-D-glucopyranoside (α,β-Trehalose)	C	143
β-D-Glucopyranosyl β-D-glucopyranoside (β,β-Trehalose)	C, D	160, 161
2-*O*-α-D-Glucopyranosyl-D-glucose (Kojibiose)	A, C, D	109, 162, 163
O-α-D-G*p*-(1→2)-*O*-[β-D-G*p*-(1→4)]-D-G*p*	D	9
2-*O*-β-D-Glucopyranosyl-D-glucose (Sophorose)	B, C, D	30, 161
O-β-D-G*p*-(1→[2-*O*-β-D-G*p*-1]$_n$→2)-D-G, n = 1–4 (Sophorodextrins)	B	164
O-β-D-G*p*-(1→2)-*O*-[β-D-G*p*-(1→3)]-D-G	C	165
3-*O*-α-D-Glucopyranosyl-D-glucose (Nigerose)	A, B, C, D	119, 121, 166, 167
O-α-D-G*p*-(1→3)-*O*-α-D-G*p*-(1→4)-D-G	B	121

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
3-*O*-β-D-Glucopyranosyl-D-glucose (Laminaribiose)	A, B, C, D	109, 168–170
O-β-D-G*p*-(1→[3-*O*-β-D-G*p*-1]$_n$→3)-D-G, n = 1–4 (Laminaridextrins)	B, C	84, 171
O-β-D-G*p*-(1→3)-*O*-β-D-G*p*-(1→4)-D-G	B	85
O-β-D-G*p*-(1→3)-*O*-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→4)-D-G	B	172
O-β-D-G*p*-(1→3)-*O*-β-D-G*p*-(1→6)-D-G	B, C	171, 173
O-β-D-G*p*-(1→3)-*O*-[β-D-G*p*-(1→6)]-D-G*p*	C	174
4-*O*-α-D-Glucopyranosyl-D-glucopyranose (Maltose)	A, B, D	98, 166, 175
O-α-D-G*p*-(1→[4-*O*-α-D-G*p*-1]$_n$→4)-D-G*p*, n = 1–4 (Maltodextrins)	A, B, D	98, 176, 177
O-α-D-G*p*-(1→[4-*O*-α-D-G*p*-1]$_n$→4)-*O* (4←1)-G*p*-D-α-*O*-(4←1)-G*p*-D-α, n = 3, 4, or 5 (Schardinger dextrins)	B	178
O-α-D-G*p*-(1→4)-*O*-α-D-G*p*(1→3)-D-G	B	121
O-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→6)-D-G (Isopanose)	D	179
O-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→4)-D-G	D	179
O-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→4)-D-G	B	35
O-α-D-G*p*-(1→4)-*O*-[β-D-G*p*-(1→6)-]-D-G*p*	C	180
4-*O*-β-D-Glucopyranosyl-D-glucopyranose (Cellobiose)	B, C, D	181–183
O-β-D-G*p*-(1→[4-*O*-β-D-G*p*-1]$_n$→4)-D-G, n = 1–5 (Cellodextrins)	B, D	106, 184
O-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→3)-D-G	B	85
O-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→3)-D-G	B	172
O-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→3)-*O*-β-D-G*p*-(1→4)-D-G	B	172
O-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→6)-D-G	C	185
5-*O*-β-D-Glucopyranosyl-D-glucofuranose	C	186
6-*O*-α-D-Glucopyranosyl-D-glucose (Isomaltose)	A, B, C, D	118, 166, 187, 188
O-α-D-G*p*-(1→[6-*O*-α-D-G*p*-1]$_n$→6)-D-G, n = 1–5 (Isomaltodextrins)	B, D	19, 58
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→3)-D-G	D	167
O-α-D-G*p*-(1→6)-*O*-β-D-G*p*-(1→3)-D-G	B	84
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→4)-D-G (Panose)	B, D	189, 190
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→4)-D-G	D	191
O-α-D-G*p*-(1→[6-*O*-α-D-G*p*-1]$_n$→6)-*O*-α-D-G*p*-(1→4)-D-G, n = 1 or 2	D	19
O-α-D-G*p*(1→6)-*O*-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→6)-D-G	D	179
6-*O*-β-D-Glucopyranosyl-D-glucopyranose (Gentiobiose)	B, C, D	161, 192, 193
O-β-D-G*p*-(1→[6-*O*-β-D-G*p*-1]$_n$→6)-D-G, n = 1–6 (Gentiodextrins)	B, C, D	80, 192, 193
O-β-D-G*p*-(1→6)-*O*-β-D-G*p*-(1→3)-D-G	B, C	171, 173
O-β-D-G*p*-(1→6)-*O*-β-D-G*p*-(1→4)-D-G	D	80

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
D-Mannose		
α-D-Mannopyranosyl α-D-mannopyranoside	C	194
α-D-Mannopyranosyl β-D-mannopyranoside	C	194
2-*O*-α-D-Mannopyranosyl-D-mannose	B, C	195, 196
O-α-D-Man*p*-(1→2)-*O*-α-D-Man*p*-(1→2)-D-Man	B	197
3-*O*-α-D-Mannopyranosyl-D-mannose	C	131
4-*O*-α-D-Mannopyranosyl-D-mannopyranose	B	198
O-α-D-Man*p*-(1→[4-*O*-β-D-Man*p*-1]$_n$→4)-D-Man*p*, n = 1–4	B	198
4-*O*-β-D-Mannopyranosyl-D-mannopyranose	B	198
O-β-D-Man*p*-(1→[4-*O*-β-D-Man*p*-1]$_n$→4)-D-Man*p*, n = 1–3	B	198
6-*O*-α-D-Mannopyranosyl-D-mannose	B, C	131
6-*O*-β-D-Mannopyranosyl-D-mannose	C	131
D-Xylose		
α-D-Xylopyranosyl α-D-xylopyranoside	C	199
3-*O*-α-D-Xylopyranosyl-D-xylose	C	199
3-*O*-β-D-Xylopyranosyl-D-xylose (Rhodymenabiose)	B, C	3, 200
4-*O*-β-D-Xylopyranosyl-D-xylopyranose (Xylobiose)	B, C	199, 201
O-β-D-Xyl*p*-(1→[4-*O*-β-D-Xyl*p*-1]$_n$→4)-D-Xyl*p*, n = 1–7	B, D	201, 202
O-β-D-Xyl*p*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*	D	3
b. Heterogeneous, Reducing Compounds		
D-Apiosyl-		
6-*O*-D-Apiosyl-D-glucose	B	96
L-Arabinosyl-		
2-*O*-β-L-Arabinopyranosyl-D-glucose	C	203
6-*O*-L-Arabinosyl-D-glucose (Vicianose)	B, C, D	204–206
O-β-L-Ara*f*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*	B	78
O-(Ara)$_n$-(Xyl)$_x$, n = 1 or 2; x = 3, 5, or 6	B	207
D-Fructosyl-		
2-*O*-β-D-Fructofuranosyl-D-glucose	D	136
3-*O*-β-D-Fructofuranosyl-D-glucose	D	136
6-*O*-β-D-Fructofuranosyl-D-glucose	D	208
2-Deoxy-6-*O*-(β-D-fructofuranosyl)-D-*arabino*-hexose	D	209
D-Fucosyl- (6-deoxy-D-galactosyl-)		
6-*O*-α-D-Fucopyranosyl-D-glucose	C	210

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
L-Fucosyl- (6-deoxy-L-galactosyl-)		
2-*O*-α-L-Fucopyranosyl-D-galactose	C	112
O-L-Fuc-(D-Gal)$_2$	C	211
O-α-L-Fuc*p*-(1→2)-*O*-β-*D*-Gal*p*-(1→4)-D-G (Fucosidolactose)	A	212
O-L-Fuc-*O*-D-Gal-*O*-L-Fuc-D-G	A	213
O-L-Fuc-*O*-D-Gal-D-G	A	213
O-L-Fucosyl-D-glucose	B	214
2-*O*-α-L-Fucopyranosyl-D-talose	C	112
D-Galactosyl-		
4-*O*-β-D-Galactopyranosyl-D-altrose (Neolactose)	C	215
3,6-Anhydro-4-*O*-(β-D-galactopyranosyl)-D-galactose (Carrabiose)	B	216
3,6-Anhydro-4-*O*-(β-D-galactopyranosyl)-L-galactose (Agarobiose)	B	217
2-*O*-D-Galactosyl-D-arabinose	C	218
3-*O*-β-D-Galactopyranosyl-D-arabinose	C, D	147, 219
5-*O*-β-D-Galactopyranosyl-D-arabinofuranose	D	147
3-*O*-α-D-Galactopyranosyl-L-arabinose	C	220
3-*O*-β-D-Galactopyranosyl-L-arabinose	B	154
5-*O*-β-D-Galactopyranosyl-L-arabinofuranose	B, C	221, 222
O-β-D-Gal*p*-(1→[6-*O*-β-D-Gal*p*-1]$_n$→3)-L-Ara, n = 1, 2, or 3	B	154
2-*O*-β-D-Galactopyranosyl-D-erythrose	C	223
4-*O*-α-D-Galactopyranosyl-D-erythrose	C	224
1-*O*-α-D-Galactopyranosyl-D-fructose	B	88
3-*O*-β-D-Galactopyranosyl-D-fructose	C	225
4-*O*-β-D-Galactopyranosyl-D-fructose (Lactulose)	A, C	226, 227
6-*O*-β-D-Galactopyranosyl-D-fructofuranose (Planteobiose)	B, C, D	87, 228, 229
O-α-D-Gal*p*-(1→6)-*O*-α-D-Gal*p*-(1→1)-D-Fru	B	88
O-α-D-Gal*p*-(1→6)-*O*-α-D-Gal*p*-(1→6)-D-Fru	C	230
O-β-D-Gal*p*-(1→6)-*O*-β-D-Gal*p*-(1→3)-D-Fru	D	231
2-*O*-α-D-Galactopyranosyl-D-glucose	B, C	203, 232
2-*O*-β-D-Galactopyranosyl-D-glucose	C	233
3-*O*-α-D-Galactopyranosyl-D-glucose	B, C	234, 235
3-*O*-β-D-Galactopyranosyl-D-glucose	C, D	156, 225
4-*O*-β-D-Galactopyranosyl-α-D-glucopyranose (α-Lactose)	A, C, D	236–238
4-*O*-β-D-Galactopyranosyl-β-D-glucopyranose (β-Lactose)	A, C, D	236–238
O-β-D-Gal*p*-(1→4)-*O*-β-D-G*p*-(1→6)-D-Gal	C	155
O-β-D-Gal*p*-(1→4)-*O*-β-D-G*p*-(1→6)-D-G*p*	C	185
O-β-D-Gal*p*-(1→4)-*O*-[-α-D-G*p*-(1→2)-]-D-G*p*	D	9
6-*O*-α-D-Galactopyranosyl-β-D-Glucopyranose (Melibiose)	A, B, C	205, 239
O-α-D-Gal*p*-(1→[6-*O*-α-D-Gal*p*-1]$_n$→6)-D-G*p*, n = 1–4 (including Manninotriose, Verbascotetraose)	A, B, D	88, 239

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
6-*O*-β-D-Galactopyranosyl-D-glucose (Allolactose)	C, D	156, 240
O-β-D-Gal*p*-(1→6)-*O*-β-D-Gal*p*-(1→4)-D-G	D	241
O-β-D-Gal*p*-(1→6)-*O*-β-D-Gal*p*-(1→6)-D-G*p*	D	156
O-Gal-*O*-(G)$_3$-*O*-(Xyl)$_2$ (Tamarindose)	B	242
3-*O*-D-Galactosyl-D-mannose	C	218
4-*O*-β-D-Galactopyranosyl-α-D-mannopyranose (α-Epilactose)	C	243
4-*O*-β-D-Galactopyranosyl-β-D-mannopyranose (β-Epilactose)	C	244
6-*O*-α-D-Galactopyranosyl-β-D-mannopyranose (Epimelibiose)	B, C, D	228, 245, 246
O-α-D-Gal*p*-(1→6)-*O*-α-D-Gal*p*-(1→6)-β-D-Man*p*	C	230
O-α-D-Gal*p*-(1→6)-*O*-β-D-Man*p*-(1→4)-D-Man*p*	B	247
4-*O*-β-D-Galactosyl-D-*ribo*-hexulose	C	248
4-*O*-β-D-Galactopyranosyl-D-xylopyranose	B	249
O-L-Gal*p*-(1→4)-*O*-D-Xyl*p*-(1→2)-L-Ara	B	111
Miscellaneous Galactosyl Derivatives		
4-*O*-β-D-*xylo*-3-Hexulosyl-D-glucose	B	250
3-*O*-(3,6-Anhydro-α-L-galactopyranosyl)-D-galactose (Neoagarobiose)	B	251
O-(3,6-Anhydro-α-L-Gal*p*)-(1→3)-*O*-β-D-Gal*p*-(1→4)-*O*-(3,6-anhydro-α-L-Gal*p*)-(1→3)-D-Gal (Neoagarotetraose)	B	252
D-Glucosyl-		
4-*O*-β-D-Glucopyranosyl-D-altrose (Celtrobiose)	C	253
2-*O*-D-Glucosyl-D-arabinose	C	254
3-*O*-α-D-Glucosyl-D-arabinose	C	255
3-*O*-β-D-Glucopyranosyl-D-arabinose	C	256
5-*O*-β-D-Glucopyranosyl-D-arabinofuranose	C	257
3-*O*-α-D-Glucopyranosyl-L-arabinopyranose	D	258
4-*O*-β-D-Glucopyranosyl-D-cymarose (Strophanthobiose, Periphlobiose)	B	259
O-D-G-*O*-D-G-*O*-D-Cym (Strophanthotriose)	B	259
2-Deoxy-3-*O*-(D-glucopyranosyl)-D-glucose	C	260
2-Deoxy-4-*O*-(α-D-glucopyranosyl)-D-*arabino*-hexose ("2-Deoxymaltose")	C	255
2-Deoxy-4-*O*-(β-D-glucopyranosyl)-D-*arabino*-hexose ("2-Deoxycellobiose")	C	261
4-*O*-β-D-Glucopyranosyl-D-digitoxose (Digilanidobiose)	B	259
O-β-D-G*p*-(1→6)-*O*-β-D-G*p*-(1→4)-D-Dig (Odorotriose)	B	262
2-*O*-α-D-Glucosyl-D-erythrose	C	224

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
2-*O*-β-D-Glucosyl-D-erythrose	C	224
1-*O*-α-D-Glucopyranosyl-D-fructose	D	47
1-*O*-β-D-Glucopyranosyl-D-fructose	C	263
3-*O*-α-D-Glucopyranosyl-D-fructose (Turanose)	A, B	166, 264
4-*O*-α-D-Glucopyranosyl-D-fructose (Maltulose)	A, C, D	47, 166, 227
O-α-D-G*p*-(1→4)-*O*-α-D-G*p*-(1→4)-D-Fru ("Maltotriulose")	C	47
4-*O*-β-D-Glucopyranosyl-D-fructose ("Cellobiulose")	C	114
5-*O*-α-D-Glucopyranosyl-D-fructopyranose (Leucrose)	D	46
6-*O*-α-D-Glucopyranosyl-D-fructofuranose ("Isomaltulose")	C, D	47, 265
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→6)-D-Fru ("Isomaltotrioulose")	C, D	47, 266
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→5)-D-Fru	D	266
2-*O*-β-D-Glucopyranosyl-D-galactose	C	196
O-β-D-G*p*-(1→2)-*O*-β-D-G*p*-(1→4)-D-Gal*p* (Lycotriose)	B	267
3-*O*-β-D-Glucopyranosyl-D-galactose (Sollabiose)	B	268
4-*O*-α-D-Glucopyranosyl-D-galactopyranose	B	269
4-*O*-β-D-Glucopyranosyl-D-galactopyranose (Lycobiose)	B	270
O-β-D-G*p*-(1→4)-*O*-β-D-G*p*-(1→6)-D-Gal	C	155
6-*O*-β-D-Glucopyranosyl-D-galactose	C	155
3-*O*-α-D-Glucosyl-D-mannose	C	254
4-*O*-α-D-Glucopyranosyl-β-D-mannopyranose	C	271
4-*O*-β-D-Glucopyranosyl-α-D-mannopyranose	B, C	272, 273
O-β-D-G*p*-(1→4)-*O*-β-D-Man*p*-(1→4)-D-Man*p*	B	69
6-*O*-β-D-Glucopyranosyl-α-D-Mannose	C	274
4-*O*-β-D-Glucopyranosyl-D-*ribo*-hexulose	C	275
4-*O*-β-D-Glucopyranosyl-L-rhamnose (Scillabiose)	B	276
O-D-G*p*-*O*-β-D-G*p*-(1→4)-L-Rha (Scillatriose)	B	277
O-D-Glucosyl-L-rhamnose (Sophorobiose)	B	278
2-*O*-β-D-Glucopyranosyl-D-xylose	C	279
O-β-D-G*p*-(1→2)-*O*[β-D-Xyl*p*-(1→3)]-*O*-β-D-G*p*-(1→4)-α-D-Gal*p* (Lycotetraose)	B	270
3-*O*-α-D-Glucopyranosyl-D-xylose	D	103
3-*O*-β-D-Glucopyranosyl-D-xylose	D	122
4-*O*-α-D-Glucopyranosyl-D-xylopyranose	D	104
D-Mannosyl-		
6-*O*-D-Mannosyl-α-D-galactose	C	155
4-*O*-β-D-Mannopyranosyl-D-glucopyranose	B	272
O-β-D-Man*p*-(1→4)-*O*-β-D-G*p*-(1→4)-D-G*p*	B	69
O-β-D-Man*p*-(1→4)-*O*-β-D-Man*p*-(1→4)-D-G*p*	B	198
6-*O*-α-D-Mannopyranosyl-D-glucose	C	210

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
L-Rhamnosyl- (6-deoxy-L-mannosyl-)		
2-*O*-α-L-Rhamnopyranosyl-D-galactose	B	268
O-α-L-Rha*p*-(1→2)-*O*-[β-D-G*p*-(1→3)]-D-galactose (Solatriose)	B	268
6-*O*-α-L-Rhamnosyl-D-galactose (Robinobiose)	B, C	210, 280
6-*O*-α-L-Rhamnosyl-D-glucose (Rutinose)	B, C	97, 210
D-Xylosyl-		
2-*O*-β-D-Xylopyranosyl-L-arabinose	B, C	281
3-*O*-α-D-Xylopyranosyl-L-arabinose	B	249
5-*O*-β-D-Xylopyranosyl-L-arabinose	B, C	282, 283
6-*O*-β-D-Xylopyranosyl-D-galactose	C	283
6-*O*-α-D-Xylopyranosyl-D-glucopyranose (Isoprimeverose)	B, C	69, 284
6-*O*-β-D-Xylopyranosyl-D-glucose (Primeverose)	B, C	285, 286
O-α-D-Xyl*p*-(1→6)-*O*-β-D-G*p*-(1→4)-D-G*p*	B	69
c. Heterogeneous, Nonreducing Compounds		
(i) Disaccharides		
D-Galactosyl-		
α-D-Galactofuranosyl α-D-glucopyranoside	D	26
α-D-Galactopyranosyl β-D-fructofuranoside	D	287
β-D-Galactopyranosyl α-D-glucopyranoside	C	160
D-Glucosyl-		
α-D-Glucopyranosyl β-D-fructofuranoside (Sucrose)	A, B, C, D	48, 288, 289
α-D-Glucopyranosyl α-L-*erythro*-pentulofuranoside	D	290
α-D-Glucopyranosyl β-D-*threo*-pentulofuranoside	D	291
α-D-Glucosyl L-lyxoside	C	196
α-D-Glucopyranosyl 6-deoxy-β-D-*arabino*-hexuloside	D	292
α-D-Glucopyranosyl α-L-*xylo*-hexulofuranoside	D	291
D-Glucosyl D-xyloside	B	293
D-Mannosyl-		
D-Mannosyl L-arabinoside	C	196
D-Xylosyl-		
α-D-Xylopyranosyl β-D-fructofuranoside	D	294
(ii) Sucrose Homologs		
***O*-D-Fructosylsucroses**		
O-β-D-Fru*f*(2→[1-*O*-β-D-Fru*f*-2]$_n$→1)-β-D-Fru*f*-(2→1) α-D-G*p*, n = 1 (Kestose, Inulobiosyl D-glucoside); n = 2 (Inulotriosyl D-glucoside)	A, D	92, 295, 296

TABLE III—*Continued*

Oligosaccharide	*Source*	*References*
O-β-D-Fru*f*-(2→[1-*O*-β-D-Fru*f*-2]$_n$→1)-*O*-[β-D-Fru*f*-(2→6)]-β-D-Fru*f*-(2→1) α-D-G*p*, n = 0 or 1	A	297
O-β-D-Fru*f*-(2→6)-β-Fru*f*-(2→1) α-D-G*p* (Kestose)	A, D	295, 298
O-β-D-Fru*f*-(2→6)-α-D-G*p*-(1→2) β-D-Fru*f* (Neokestose)	A, D	295, 299
***O*-D-Galactosylsucroses**		
O-α-D-Gal*p*-(1→2)-α-D-G*p*-(1→2) β-D-Fru*f* (Umbelliferose)	A	300
O-α-D-Gal*p*-(1→3)-β-D-Fru*f*-(2→1) α-D-G*p*	A	301
O-α-D-Gal*p*-(1→3)-α-D-G*p*-(1→2) β-D-Fru*f*	A	234
O-D-Gal-(1→[4-*O*-D-Gal-1]$_3$→4)-D-Gal(1→2) D-Fru (Lycopose)	A	302
O-β-D-Gal*p*-(1→4)-α-D-G*p*-(1→2) β-D-Fru*f* (Lactosylsucrose)	D	303
O-α-D-Gal*p*-(1→6)-β-D-Fru*f*-(2→1) α-D-G*p* (Planteose)	A	239
O-α-D-Gal*p*-(1→6)-α-D-G*p*-(1→2) β-D-Fru*f* (Raffinose)	A, B, D	239, 304
O-α-D-Gal*p*-(1→[6-*O*-α-D-Gal*p*-1]$_n$→6)-α-D-G*p*-(1→2) β-D-Fru*f*, n = 1 (Stachyose); n = 2 (Verbascose); n = 3(Ajugose); n = 4 or 5.	A	88, 239
O-α-D-Gal*p*-(1→6)-*O*-[α-D-G*p*-(1→2)]-α-D-G*p*-(1→2) β-D-Fru*f*	D	305
O-α-D-Gal*p*-(1→[6-*O*-α-D-Gal*p*-1]$_n$→6)-*O*-α-D-Gal*p*-(1→1)-*O*-[αD-Gal*p*-(1→6)-α-D-G*p*-(1→2)] β-D-Fru*f*, n = 0 (Lychnose), 1, or 2	A	88
O-α-D-Gal*p*-(1→[6-*O*-α-D-Gal*p*-1]$_n$→6)-*O*-α-D-Gal*p*-(1→3)-*O*[α-D-Gal*p*-(1→6)-*O*-α-D-G*p*-(1→2)-] β-D-Fru*f*, n = 0 (Isolychnose), 1, or 2	A	88
***O*-D-Glucosylsucroses**		
O-α-D-G*p*-(1→3)-β-D-Fru*f*-(2→1) α-D-G*p* (Melezitose)	A, D	306
O-α-D-G*p*-(1→4)-α-D-G*p*-(1→2) β-D-Fru*f* (Erlose)	A, D	306, 307
O-α-D-G*p*-(1→[4-*O*-α-D-G*p*-1]$_n$→4)-α-D-G*p*-(1→2) β-D-Fru*f*, n = 0 (Maltosylsucrose); n = 1 (Maltotriosylsucrose)	A, D	107, 308
O-α-D-G*p*-(1→6)-α-D-G*p*-(1→2) β-D-Fru*f*	D	102
O-β-D-G*p*-(1→6)-α-D-G*p*-(1→2) β-D-Fru*f* (Gentianose)	A	309

(128) A. B. Foster and D. Horton, *Advances in Carbohydrate Chem.*, **14**, 213 (1959).
(129) F. A. H. Rice, *J. Am. Chem. Soc.*, **78**, 6167 (1956).
(130) P. Andrews, L. Hough, and D. B. Powell, *Chem. & Ind.* (London), 658 (1956).
(131) J. K. N. Jones and W. H. Nicholson, *J. Chem. Soc.*, 27 (1958).
(132) P. Andrews and J. K. N. Jones, *J. Chem. Soc.*, 4134 (1954).
(133) A. M. Stephen, *J. Chem. Soc.*, 1919 (1957).
(134) S. M. Strepkov, *Doklady Akad. Nauk S. S. S. R.*, **124**, 1344 (1959); *Chem. Abstracts*, **53**, 21686 (1959).
(135) J. H. Pazur and A. L. Gordon, *J. Am. Chem. Soc.*, **75**, 3458 (1953).
(136) D. S. Feingold, G. Avigad, and S. Hestrin, *Biochem. J.*, **64**, 351 (1956).

(137) H. H. Schlubach and G. Blaschke, *Ann.*, **595**, 224 (1955).
(138) R. Zelikson and S. Hestrin, *Biochem. J.*, **79**, 71 (1961).
(139) R. H. Côté, *J. Chem. Soc.*, 2248 (1959).
(140) M. J. Clancy and W. J. Whelan, *Biochem. J.*, **76**, 22P (1960).
(141) P. A. J. Gorin and J. F. T. Spencer, *Can. J. Chem.*, **37**, 499 (1959).
(142) H. Bredereck, G. Höschele, and K. Ruck, *Chem. Ber.*, **86**, 1277 (1953).
(143) V. E. Sharp and M. Stacey, *J. Chem. Soc.*, 285 (1951).
(144) K. Morgan and A. N. O'Neill, *Can. J. Chem.*, **37**, 1201 (1959).
(145) G. O. Aspinall, E. L. Hirst, and E. Ramstad, *J. Chem. Soc.*, 593 (1958).
(146) D. H. Ball and J. K. N. Jones, *J. Chem. Soc.*, 905 (1958).
(147) J. H. Pazur, M. Shadaksharaswamy, and A. Cepure, *Arch. Biochem. Biophys.*, **94**, 142 (1961).
(148) R. L. Whi tler and H. E. Conrad, *J. Am. Chem. Soc.*, **76**, 1673 (1954).
(149) J. K. Gillham, A. S. Perlin, and T. E. Timell, *Can. J. Chem.*, **36**, 1741 (1958).
(150) H. Masamune and S. Kamiyama, *Tôhoku J. Exptl. Med.*, **66**, 43 (1957); *Chem. Abstracts*, **52**, 8974 (1958).
(151) H. O. Bouveng and H. Muir, *Acta Chem. Scand.*, **13**, 1884 (1959).
(152) T. R. Ingle and B. V. Bhide, *J. Indian Chem. Soc.*, **35**, 516 (1958).
(153) C. N. Turton, A. Bebbington, S. Dixon, and E. Pacsu, *J. Am. Chem. Soc.*, **77**, 2565 (1955).
(154) G. O. Aspinall, B. J. Auret, and E. L. Hirst, *J. Chem. Soc.*, 4408 (1958).
(155) K. Freudenberg, A. Wolf, E. Knopf, and S. H. Zaheer, *Ber.*, **61**, 1743 (1928).
(156) J. H. Pazur, C. L. Tipton, T. Budovitch, and J. M. Marsh, *J. Am. Chem. Soc.*, **80**, 119 (1958).
(157) L. C. Stewart, N. K. Richtmyer, and C. S. Hudson, *J. Am. Chem. Soc.*, **72**, 2059 (1950).
(158) R. U. Lemieux and H. F. Bauer, *Can. J. Chem.*, **32**, 340 (1954).
(159) E. Cabib and L. F. Leloir, *J. Biol. Chem.*, **231**, 259 (1958).
(160) B. Helferich and K. Weis, *Chem. Ber.*, **89**, 314 (1956).
(161) S. Peat, W. J. Whelan, and K. A. Hinson, *Nature*, **170**, 1056 (1952).
(162) T. Watanabe and K. Aso, *Nature*, **183**, 1740 (1959).
(163) K. Aso, K. Shibasaki, and M. Nakamura, *Nature*, **182**, 1303 (1958).
(164) P. A. J. Gorin, J. F. T. Spencer, and D. W. S. Westlake, *Can. J. Chem.*, **39**, 1067 (1961).
(165) A. Klemer and K. Homberg, *Chem. Ber.*, **93**, 1643 (1960).
(166) J. W. White and N. Hoban, *Arch. Biochem. Biophys.*, **80**, 386 (1959).
(167) J. H. Pazur, T. Budovitch, and C. L. Tipton, *J. Am. Chem. Soc.*, **79**, 625 (1957).
(168) T. Nagahama, K. Nishida, and T. Numata, *Mem. Fac. Agr. Kagoshima Univ.*, **4**, 9 (1960); *Chem. Abstracts*, **55**, 703 (1961).
(169) J. J. Connell, E. L. Hirst, and E. G. V. Percival, *J. Chem. Soc.*, 3494 (1950).
(170) P. Bächli and E. G. V. Percival, *J. Chem. Soc.*, 1243 (1952).
(171) S. Peat, W. J. Whelan, and J. M. Evans, *J. Chem. Soc.*, 175 (1960).
(172) F. W. Parrish, A. S. Perlin, and E. T. Reese, *Can. J. Chem.*, **38**, 2094 (1960).
(173) S. Peat, W. J. Whelan, and T. E. Edwards, *J. Chem. Soc.*, 3862 (1958).
(174) J. R. Turvey and J. M. Evans, *J. Chem. Soc.*, 2366 (1960).
(175) C. Fitting and M. Doudoroff, *J. Biol. Chem.*, **199**, 153 (1952).
(176) W. H. Fishman and H. Sie, *J. Am. Chem. Soc.*, **80**, 121 (1958).
(177) S. A. Barker and E. J. Bourne, *J. Chem. Soc.*, 209 (1952).
(178) D. French, *Advances in Carbohydrate Chem.*, **12**, 189 (1957).
(179) J. H. Pazur and T. Ando, *J. Biol. Chem.*, **235**, 297 (1960).

(180) A. Klemer, *Angew. Chem.*, **69,** 638 (1957).
(181) G. Braun, *Org. Syntheses, Coll. Vol.* **2,** 122 (1943).
(182) W. T. Haskins, R. M. Hann, and C. S. Hudson, *J. Am. Chem. Soc.*, **64,** 1289 (1942).
(183) C. J. Sih, N. M. Nelson, and R. H. McBee, *Science*, **126,** 1116 (1957).
(184) H. W. Buston and M. Jabbar, *Biochim. et Biophys. Acta*, **15,** 543 (1954).
(185) B. Helferich and W. Schafer, *Ann.*, **450,** 229 (1926).
(186) K. Freudenberg and K. van Oertzen, *Ann.*, **574,** 37 (1951).
(187) M. L. Wolfrom, A. O. Pittet, and I. C. Gillham, *Proc. Natl. Acad. Sci. U. S.*, **47,** 700 (1961).
(188) S. A. Barker and T. R. Carrington, *J. Chem. Soc.*, 3588 (1954).
(189) A. Thompson and M. L. Wolfrom, *J. Am. Chem. Soc.*, **73,** 5849 (1951).
(190) M. Killey, R. J. Dimler, and J. E. Cluskey, *J. Am. Chem. Soc.*, **77,** 3315 (1955).
(191) W. A. M. Duncan and D. J. Manners, *Biochem. J.*, **69,** 343 (1958).
(192) B. Lindberg and J. McPherson, *Acta Chem. Scand.*, **8,** 985 (1954).
(193) S. Haq and W. J. Whelan, *J. Chem. Soc.*, 4543 (1956).
(194) F. Micheel and D. Bormann, *Chem. Ber.*, **93,** 1143 (1960).
(195) P. A. J. Gorin and A. S. Perlin, *Can. J. Chem.*, **34,** 1796 (1956).
(196) A. M. Gakhokidze and N. D. Kutidze, *Zhur. Obshcheĭ Khim.*, **22,** 139, 247 (1952).
(197) P. A. J. Gorin and A. S. Perlin, *Can. J. Chem.*, **35,** 262 (1957).
(198) G. O. Aspinall, R. B. Rashbrook, and G. Kessler, *J. Chem. Soc.*, 215 (1958).
(199) D. H. Ball and J. K. N. Jones, *J. Chem. Soc.*, 33 (1958).
(200) E. J. C. Curtis and J. K. N. Jones, *Can. J. Chem.*, **38,** 1305 (1960).
(201) R. L. Whistler and C.-C. Tu, *J. Am. Chem. Soc.*, **74,** 3609 (1952); **75,** 645 (1953).
(202) B. H. Howard, G. Jones, and M. R. Purdom, *Biochem. J.*, **74,** 173 (1960).
(203) J. Lehmann and D. Beck, *Ann.*, **630,** 56 (1960).
(204) G. Bertrand and G. Weisweiller, *Compt. rend.*, **150,** 180 (1910).
(205) B. Helferich and H. Bredereck, *Ann.*, **465,** 166 (1928).
(206) K. Wallenfels and D. Beck, *Ann.*, **630,** 46 (1960).
(207) C. T. Bishop and D. R. Whitaker, *Chem. & Ind.* (London), 119 (1955).
(208) D. J. Bell and J. Edelman, *J. Chem. Soc.*, 4652 (1954).
(209) G. A. Barber, *J. Am. Chem. Soc.*, **81,** 3722 (1959).
(210) P. A. J. Gorin and A. S. Perlin, *Can. J. Chem.*, **37,** 1930 (1959).
(211) H. Masamune and H. Shinohara, *Tôhoku J. Exptl. Med.*, **69,** 53 (1958); *Chem. Abstracts*, **53,** 19007 (1959).
(212) R. Kuhn, H. H. Baer, and A. Gauhe, *Chem. Ber.*, **89,** 2513, 2514 (1956).
(213) J. Montreuil, *Compt. rend.*, **242,** 192, 828 (1956).
(214) R. G. Eagon and R. Dedonder, *Compt. rend.*, **241,** 579 (1955).
(215) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **57,** 1716 (1935).
(216) T. J. Painter, *Chem. & Ind.* (London), 1488 (1959).
(217) C. Araki, *Proc. Intern. Congr. Biochem., 4th Congr. Vienna*, 1958, **1,** 15 (1959).
(218) A. M. Gakhokidze and E. E. Kobiashvili, *Zhur. Obshcheĭ Khim.*, **22,** 244 (1952).
(219) H. L. Frush and H. S. Isbell, *J. Research Natl. Bur. Standards*, **50,** 133 (1953).
(220) A. J. Charlson, J. R. Nunn, and A. M. Stephen, *J. Chem. Soc.*, 269 (1955).
(221) H. C. Srivastava and F. Smith, *J. Am. Chem. Soc.*, **79,** 982 (1957).
(222) I. J. Goldstein, F. Smith, and H. C. Srivastava, *J. Am. Chem. Soc.*, **79,** 3858 (1957).
(223) A. M. Gakhokidze, *Zhur. Obshcheĭ Khim.*, **20,** 116 (1950).
(224) A. J. Charlson and A. S. Perlin, *Can. J. Chem.*, **34,** 1200 (1956).

(225) R. Kuhn and H. H. Baer, *Chem. Ber.*, **87**, 1560 (1954).
(226) S. Adachi, *Nature*, **181**, 840 (1958).
(227) W. M. Corbett and J. Kenner, *J. Chem. Soc.*, 1789 (1954).
(228) L. Hough, J. K. N. Jones, and E. L. Richards, *J. Chem. Soc.*, 295 (1954).
(229) D. French, R. J. Suhadolnik, and L. A. Underkofler, *Science*, **117**, 100 (1953).
(230) A. de Grandchamp-Chaudin, J. E. Courtois, and P. L. Dizet, *Bull. soc. chim. biol.*, **42**, 227 (1960).
(231) A. Ballio and S. Russi, *J. Chromatog.*, **4**, 117 (1960).
(232) A. Wickstrøm, *Acta Chem. Scand.*, **11**, 1473 (1957).
(233) A. M. Gakhokidze, *Soobshcheniya Akad. Nauk Gruzin. S. S. R.*, **9**, 561 (1948); *Chem. Abstracts*, **50**, 10657 (1956).
(234) A. M. Macleod and H. McCorquodale, *Nature*, **182**, 815 (1958).
(235) A. M. Gakhokidze, *Soobshcheniya Akad. Nauk Gruzin. S. S. R.*, **10**, 85 (1949); *Chem. Abstracts*, **47**, 6875 (1953).
(236) "Richmonds Dairy Chemistry," J. G. Davies and F. J. McDonald, eds., C. Griffin, London, Engl. (1953).
(237) E. J. C. Curtis and J. K. N. Jones, *Can. J. Chem.*, **37**, 358 (1959).
(238) J. E. Gander, W. E. Petersen, and P. D. Boyer, *Arch. Biochem. Biophys.*, **69**, 85 (1957).
(239) D. French, *Advances in Carbohydrate Chem.*, **9**, 149 (1954).
(240) B. Helferich and G. Sparmberg, *Ber.*, **66**, 806 (1933).
(241) A. Ballio and S. Russi, *Tetrahedron*, **9**, 125 (1960).
(242) P. S. Rao and R. M. Beri, *Proc. Indian Acad. Sci.*, **35A**, 1 (1952).
(243) W. N. Haworth, E. L. Hirst, M. M. T. Plant, and R. J. W. Reynolds, *J. Chem. Soc.*, 2644 (1930).
(244) W. T. Haskins, R. M. Hann, and C. S. Hudson, *J. Am. Chem. Soc.*, **64**, 1852 (1942).
(245) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **73**, 4189 (1951).
(246) J. E. Courtois and F. Petek, *Bull. soc. chim. biol.*, **39**, 715 (1957).
(247) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **74**, 5140 (1952).
(248) J. P. L. Bots, *Rec. trav. chim.*, **76**, 515 (1957).
(249) R. Montgomery, F. Smith, and H. C. Srivastava, *J. Am. Chem. Soc.*, **79**, 698 (1957).
(250) M. J. Bernaerts and J. de Ley, *Biochim. et Biophys. Acta*, **30**, 661 (1958).
(251) C. Araki and K. Arai, *Bull. Chem. Soc. Japan*, **29**, 339 (1956); *Chem. Abstracts*, **51**, 3465 (1957).
(252) C. Araki and K. Arai, *Bull. Chem. Soc. Japan*, **30**, 287 (1957); *Chem. Abstracts*, **51**, 18027 (1957).
(253) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **58**, 2534 (1936).
(254) A. M. Gakhokidze and I. A. Gvelukashvili, *Zhur. Obshcheĭ Khim.*, **22**, 143 (1952).
(255) A. M. Gakhokidze, *Zhur. Obshcheĭ Khim.*, **18**, 60 (1948).
(256) G. Zemplén, *Ber.*, **59**, 1254 (1926).
(257) N. S. McDonald and W. L. Evans, *J. Am. Chem. Soc.*, **64**, 2731 (1942).
(258) W. Z. Hassid, M. Doudoroff, A. L. Potter, and H. A. Barker, *J. Am. Chem. Soc.*, **70**, 306 (1948).
(259) H. Lichli and A. von Wartburg, *Helv. Chim. Acta*, **44**, 238 (1961).
(260) A. M. Gakhokidze, *Zhur. Obshcheĭ Khim.*, **19**, 2082 (1949).
(261) A. M. Gakhokidze, *Zhur. Obshcheĭ Khim.*, **16**, 1914 (1946).
(262) W. Rittel and T. Reichstein, *Helv. Chim. Acta*, **37**, 1361 (1954).
(263) E. Pacsu, E. J. Wilson, and L. Graf, *J. Am. Chem. Soc.*, **61**, 2675 (1939).

(264) C. S. Hudson and E. S. Pacsu, *J. Am. Chem. Soc.*, **52**, 2519 (1930).
(265) E. S. Sharpe, F. H. Stodola, and H. J. Koepsell, *J. Org. Chem.*, **25**, 1060 (1960).
(266) E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, **79**, 549 (1961).
(267) R. Kuhn, I. Löw, and H. Trischmann, *Angew. Chem.*, **68**, 212 (1958).
(268) R. Kuhn, I. Löw, and H. Trischmann, *Chem. Ber.*, **88**, 1492 (1955).
(269) J. K. N. Jones and M. B. Perry, *J. Am. Chem. Soc.*, **79**, 2787 (1957).
(270) R. Kuhn, I. Löw, and H. Trischmann, *Chem. Ber.*, **90**, 203 (1957).
(271) W. N. Haworth, E. L. Hirst, and R. J. W. Reynolds, *J. Chem. Soc.*, 302 (1934).
(272) F. Smith and H. C. Srivastava, *J. Am. Chem. Soc.*, **78**, 1404 (1956).
(273) W. N. Haworth, E. L. Hirst, H. R. L. Streight, H. A. Thomas, and J. I. Webb, *J. Chem. Soc.*, 2636 (1930).
(274) H. J. Dauben and W. L. Evans, *J. Am. Chem. Soc.*, **60**, 886 (1938).
(275) L. Hough, J. K. N. Jones, and E. L. Richards, *J. Chem. Soc.*, 2005 (1953).
(276) G. Zemplén, *Math. u. naturw. Anz. ungar. Akad. Wiss.*, **57**, 999 (1938); *Chem. Abstracts*, **33**, 4202 (1939).
(277) A. Stoll, W. Kreiss, and A. von Wartburg, *Helv. Chim. Acta*, **35**, 2495 (1952).
(278) G. Zemplén and R. Bognár, *Ber.*, **75**, 482 (1942).
(279) J. K. N. Jones and P. E. Reid, *Can. J. Chem.*, **38**, 944 (1960).
(280) G. Zemplén and A. Geracs, *Ber.*, **68**, 2054 (1935).
(281) G. O. Aspinall and R. J. Ferrier, *Chem. & Ind.* (London), 819 (1957).
(282) P. Andrews, D. H. Ball, and J. K. N. Jones, *J. Chem. Soc.*, 4090 (1953).
(283) D. H. Ball and J. K. N. Jones, *J. Chem. Soc.*, 4871 (1957).
(284) G. Zemplén, and R. Bognár, *Ber.*, **72**, 1160 (1939).
(285) R. Hill and D. Richter, *J. Chem. Soc.*, 1714 (1936).
(286) B. Helferich and H. Rauch, *Ann.*, **455**, 168 (1927).
(287) D. S. Feingold, G. Avigad, and S. Hestrin, *J. Biol. Chem.*, **224**, 295 (1957).
(288) R. U. Lemieux and G. Huber, *J. Am. Chem. Soc.*, **78**, 4117 (1956).
(289) C. E. Cardini, L. F. Leloir, and J. Chiriboga, *J. Biol. Chem.*, **214**, 149 (1955).
(290) M. Doudoroff, W. Z. Hassid, and H. A. Barker, *J. Biol. Chem.*, **168**, 733 (1947).
(291) W. Z. Hassid, M. Doudoroff, H. A. Barker, and W. H. Dore, *J. Am. Chem. Soc.*, **67**, 1394 (1945); **68**, 1465 (1946).
(292) N. J. Palleroni and M. Doudoroff, *J. Biol. Chem.*, **219**, 957 (1956).
(293) F. B. Power and A. H. Salway, *J. Chem. Soc.*, **105**, 1062 (1914).
(294) G. Avigad, D. S. Feingold, and S. Hestrin, *Biochim. et Biophys. Acta*, **20**, 129 (1956).
(295) P. J. Allen and J. S. D. Bacon, *Biochem. J.*, **63**, 200 (1956).
(296) J. H. Pazur, *J. Biol. Chem.*, **199**, 217 (1952).
(297) H. H. Schlubach and H. O. Koehn, *Ann.*, **614**, 126 (1958).
(298) N. Albon, D. J. Bell, P. H. Blanchard, D. Gross, and J. T. Rundell, *J. Chem. Soc.*, 24 (1953).
(299) J. S. D. Bacon, *Biochem. J.*, **73**, 507 (1959).
(300) A. Wickstrøm and A. B. Svendsen, *Acta Chem. Scand.*, **10**, 1119 (1956).
(301) J. E. Courtois, P. L. Dizet, and F. Petek, *Bull. soc. chim. biol.*, **41**, 1261 (1959).
(302) S. Murakami, *Acta Phytochim.* (Japan), **13**, 37 (1942).
(303) G. Avigad, *J. Biol. Chem.*, **229**, 121 (1957).
(304) S. Hestrin, D. S. Feingold, and G. Avigad, *J. Am. Chem. Soc.*, **77**, 6710 (1955).
(305) W. B. Neely, *Arch. Biochem. Biophys.*, **79**, 154 (1959).
(306) J. S. D. Bacon and B. Dickinson, *Biochem. J.*, **66**, 289 (1957).
(307) J. W. White and J. Maher, *J. Am. Chem. Soc.*, **75**, 1259 (1953).
(308) J. P. Wolf and W. H. Ewart, *Arch. Biochem. Biophys.*, **58**, 365 (1955).
(309) M. Bridel and M. Desmarest, *J. pharm. chim.*, **9**, 465 (1929).

TABLE IV

Compounds Containing Amino Sugars

a. Amino Sugars Plus Simple Monosaccharides[a]

Oligosaccharide	*Source*	*References*
Containing 2-Amino-2-deoxy-D-galactose		
2-Acetamido-2-deoxy-6-*O*-(α-D-galactopyranosyl)-D-galactose	D	310
3-*O*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose	B	311
O-α-D-Gal*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→3)-D-GNAc	B	312
O-α-D-Gal*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-D-GNAc	B	312
O-α-D-Gal*p*NAc (1→4)-*O*-β-D-Gal*p*-(1→4)-D-GNAc	B	313
2-Acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyl)-α-D-galactopyranose	B	314
Containing 2-Amino-2-deoxy-D-glucose		
2-Acetamido-2-deoxy-6-*O*-(L-fucopyranosyl)-D-glucose	B	311
O-α-L-Fuc*p*-(1→2)-*O*-β-D-Gal*p*-(1→3)-*O*-β-D-G*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-D-G*p* (Lacto-*N*-fucopentaose I)	A	212
O-α-L-Fuc*p*-(1→4)-*O*-[β-D-Gal*p*-(1→3)]-*O*-β-D-G*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-D-G*p* (Lacto-*N*-fucopentaose II)	A	315
Mono-*O*-L-fucosyl-(lacto-*N*-tetraose)$_n$, n = 2 or 3	A	316
O-α-L-Fuc*p*-(1→4)-*O*-[β-D-Gal*p*-(1→3)]-*O*-β-D-G*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-*O*-[α-L-Fuc*p*-(1→3)]-D-G*p* (Lacto-*N*-difucohexaose, Difuco-lacto-*N*-tetraose)	A	317
Di-*O*-L-fucosyl-(lacto-*N*-tetraose)$_n$, n = 2 or 3	A	316
(L-Fuc)$_2$-D-G-D-GNAc	B	214
2-Acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-glucose (Lacto-*N*-biose I)	B, D	318, 319
O-β-D-Gal*p*-1(1→3)-*O*-β-D-G*p*NAc-(1→3)-D-Gal (Lacto-*N*-triose I)	B	320
O-β-D-Gal*p*-(1→3)-*O*-β-D-G*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-D-G*p* (Lacto-*N*-tetraose)	A	321
(Lacto-*N*-tetraose)$_2$	A	316
2-Acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-D-glucopyranose (*N*-Acetyllactosamine)	B, C, D	311, 319, 322
2-Acetamido-2-deoxy-6-*O*-(α-D-galactopyranosyl)-D-glucose	D	310
2-Acetamido-2-deoxy-6-*O*-(β-D-galactopyranosyl)-D-glucose	C, D	319, 323
3-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-galactose (Lacto-*N*-biose II)	B	311
O-β-D-G*p*NAc-(1→3)-*O*-β-D-Gal*p*-(1→4)-D-G (Lacto-*N*-triose II)	B	320

TABLE IV—*Continued*

Oligosaccharide	*Source*	*References*
6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-galactose	C	324
α-D-Glucopyranosyl 2-amino-2-deoxy-α-D-glucopyranoside (Trehalosamine)	A	325
O-α-D-Glucopyranosyl-(1→4)-2-amino-2-deoxy-α-D-glucopyranose·HCl	B, C	125a
O-(2-Amino-2-deoxy-α-D-glucopyranosyl·HCl)-(1→4)-α-D-glucopyranose	B, C	326
2-Acetamido-2-deoxy-4-*O*-(β-D-glucopyranosyl)-D-glucose	B	55a
6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucose	C	324
2-Acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (Chitobiose)	B	54
O-β-D-G*p*NAc-(1→[4-*O*-β-D-G*p*NAc-1]$_n$→4)-D-G*p*NAc, n = 1–6 (Chitodextrins)	B	54
O-β-D-G*p*N-(1→[4-*O*-β-D-G*p*N-1]$_n$→4)-D-G*p*N, n = 0–3	C	327
2-Acetamido-6-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-2-deoxy-D-glucose	C	53
2-Acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose	C	53
2-Deoxy-2-(D-mannosylamino)-D-glucose·HCl	C	328
2-Amino-2-deoxy-6-*O*-(D-mannosyl)-D-glucose·HCl	C	328
Diaminohexosamine		
2-*O*-(2,6-Diamino-2,6-dideoxyhexosyl)-D-ribopyranose (Neobiosamine C)	B	329
3-*O*-(2,6-Diamino-2,6-dideoxyhexosyl)-D-ribose (Paramobiosamine, neobiosamine B)	B	330, 331
b. Amino Sugars Plus Uronic Acids		
Containing 2-Amino-2-deoxy-D-galactose		
2-Acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (Chondrosine)	B	332
2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose	B	333
2-Acetamido-2-deoxy-*O*-(D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose	B	333
Containing 2-Amino-2-deoxy-D-glucose		
O-D-G*p*NAc-*O*-β-D-G*p*A-(1→3)-D-GNAc	B	334
O-D-G*p*NAc-*O*-D-G*p*A-*O*-D-GNAc-D-GA	B	335
2-Amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (Hyalobiouronic acid)	B	126
2-Amino-2-deoxy-6-*O*-(D-glucopyranosyluronic acid)-D-glucose	B	125

TABLE IV—*Continued*

Oligosaccharide	*Source*	*References*
O-D-G*p*A-*O*-D-G*p*NAc-D-GA	B	335
O-D-G*p*A-[*O*-α-D-G*p*NAc-(1→3)-*O*-D-G*p*A-]$_{n-1}$-D-GNAc, n = 1–7	B, D	336, 337
2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose	B	338

[a] Foster and Horton[128] tabulate full details for 17 disaccharides containing amino sugars.

(310) W. M. Watkins, *Nature*, **181**, 117 (1958).
(311) R. H. Côté and W. T. J. Morgan, *Nature*, **178**, 1171 (1956).
(312) I. A. F. L. Cheese and W. T. J. Morgan, *Nature*, **191**, 149 (1961).
(313) H. Masamune and H. Shinohara, *Tôhoku J. Exptl. Med.*, **69**, 65 (1958); *Chem. Abstracts*, **53**, 19007 (1959).
(314) M. L. Wolfrom and B. O. Juliano, *J. Am. Chem. Soc.*, **82**, 1673 (1960).
(315) R. Kuhn, H. H. Baer, and A. Gauhe, *Chem. Ber.*, **91**, 364 (1958).
(316) F. H. Malpress and F. E. Hytten, *Biochem. J.*, **68**, 708 (1958).
(317) R. Kuhn and A. Gauhe, *Chem. Ber.*, **93**, 647 (1960).
(318) R. Kuhn, H. H. Baer, and A. Gauhe, *Chem. Ber.*, **87**, 1553 (1954).
(319) A. Alessandrini, E. Schmidt, F. Zilliken, and P. György, *J. Biol. Chem.*, **220**, 71 (1956).
(320) R. Kuhn, A. Gauhe, and H. H. Baer, *Chem. Ber.*, **89**, 1027 (1956).
(321) R. Kuhn and H. H. Baer, *Chem. Ber.*, **89**, 504 (1956).
(322) R. Kuhn, W. Kirschenlohr, and W. Bister, *Ann.*, **600**, 135 (1956).
(323) R. Kuhn, H. H. Baer, and A. Gauhe, *Chem. Ber.*, **88**, 1713 (1955).
(324) R. Kuhn and W. Kirschenlohr, *Chem. Ber.*, **87**, 384 (1954).
(325) F. Arcamone and F. Bizioli, *Gazz. chim. ital.*, **87**, 896 (1957).
(326) M. L. Wolfrom, J. R. Vercellotti, and D. Horton, in press.
(327) H. P. Lenk, M. W. Wenzel, and E. Schutte, *Naturwissenschaften*, **47**, 516 (1960).
(328) S. A. Barker, K. Murray, M. Stacey, and D. B. E. Stroud, *Nature*, **191**, 142, 143 (1961).
(329) K. L. Rinehart and P. K. Woo, *J. Am. Chem. Soc.*, **80**, 6463 (1958).
(330) T. M. Haskell, J. C. French, and Q. R. Bartz, *J. Am. Chem. Soc.*, **81**, 3481, 3482 (1959).
(331) K. L. Rinehart, A. D. Argoudelis, T. P. Culbertson, W. S. Chilton, and K. Streigler, *J. Am. Chem. Soc.*, **82**, 2970 (1960).
(332) E. A. Davidson and K. Meyer, *J. Am. Chem. Soc.*, **77**, 4796 (1955).
(333) S. Suzuki, *J. Biol. Chem.*, **235**, 3580 (1960).
(334) A. Linker, K. Meyer, and B. Weissmann, *J. Biol. Chem.*, **213**, 237 (1955).
(335) A. Linker, P. Hoffman, and K. Meyer, *Nature*, **180**, 810 (1957).
(336) B. Weissmann, K. Meyer, P. Sampson, and A. Linker, *J. Biol. Chem.*, **208**, 417 (1954).
(337) B. Weissmann, *J. Biol. Chem.*, **216**, 783 (1955).
(338) A. Linker, K. Meyer, and P. Hoffman, *J. Biol. Chem.*, **219**, 13 (1956).

TABLE V

Compounds Containing Uronic Acids

a. Uronic Acid Plus Simple Monosaccharide

Oligosaccharide	*Source*	*References*
Containing L-Arabinuronic acid		
6-*O*-L-Arabinofuranosyluronic acid-D-galactose	B	339
3-*O*-L-Arabinofuranosyluronic acid-D-xylose	B	49
Containing D-Galacturonic Acid		
3-*O*-D-Galactopyranosyluronic acid-D-galactose	B	340
6-*O*-β-D-Galactopyranosyluronic acid-D-galactose	B	339
4-*O*-α-D-Galactopyranosyluronic acid-D-galactopyranosyluronic acid	B	341
O-α-D-Gal*p*A-(1→[4-*O*-α-D-Gal*p*A-1]$_n$-4)-D-Gal*p*A, n = 1 or 2	B	342, 343
2-*O*-D-Galactopyranosyluronic acid-L-rhamnose	B	344
O-D-GalA-(1→2)-*O*-L-Rha-(1→4)-D-Gal	B	345
4-*O*-α-D-Galactopyranosyluronic acid-D-xylopyranose	B	346
Containing D-Glucuronic Acid		
2-*O*-α-D-Glucopyranosyl-D-glucuronic acid	D	115
O-α-D-G*p*-(1→6)-*O*-α-D-G*p*-(1→2)-D-GA	D	115
3-*O*-α-D-Glucopyranosyluronic acid-D-galactose	B	347
4-*O*-α-D-Glucosyluronic acid-D-galactose	B	348
6-*O*-β-D-Glucopyranosyluronic acid-D-galactose	B, C	220, 349
O-β-D-G*p*A-(1→6)-*O*-D-Gal*p*-D-Gal	B	350
4-*O*-β-D-Glucopyranosyluronic acid-D-glucopyranose (Cellobiouronic acid)	B, C	269, 351
O-β-D-G*p*A-(1→4)-*O*-β-D-G*p*-(1→4)-D-G*p*	B	269
O-β-D-G*p*A-(1→4)-*O*-β-D-G*p*-(1→4)-*O*-α-D-Gp-(1→4)-D-Gal*p*	B	269
2-*O*-β-D-Glucopyranosyluronic acid-D-mannopyranose	B	352
4-*O*-α-D-Glucopyranosyluronic acid-D-mannopyranose	B	50
2-*O*-α-D-Glucopyranosyluronic acid-L-rhamnose	B	353
4-*O*-β-D-Glucopyranosyluronic acid-L-rhamnose	B	354
2-*O*-α-D-Glucopyranosyluronic acid-D-xylose	B	355
2-*O*-β-D-Glucopyranosyluronic acid-D-xylopyranose	C	356
3-*O*-α-D-Glucopyranosyluronic acid-D-xylopyranose	B	357
3-*O*-β-D-Glucopyranosyluronic acid-D-xylose	B	358
4-*O*-α-D-Glucopyranosyluronic acid-D-xylose	B	359
O-α-D-G*p*A-(1→4)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*	B	360
D-Guluronic Acid		
D-Gulosyluronic acid-D-mannuronic acid$_n$, n = 1 or 2	B	361

TABLE V—*Continued*

Oligosaccharide	*Source*	*References*
D-Mannuronic Acid		
4-*O*-α-D-Mannopyranosyluronic acid-D-glucopyranose	B	50
4-*O*-β-D-Mannopyranosyluronic acid-α-D-mannopyranuronic acid	C	362
b. Monomethyl Uronic Acids Plus Simple Monosaccharides		
3-*O*-(3-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose	B	363
4-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-L-arabinose	B	364
4-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-galactose	B	365
6-*O*-(4-*O*-Methyl-β-D-glucopyranosyluronic acid)-D-galactose	B	365
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-lyxose	C	366
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose	B	367
O-(4-*O*-Me-α-D-G*p*A)-(1→2)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*	B	367
O-(4-*O*-Me-α-D-G*p*A)-(1→2)-[*O*-β-D-Xyl*p*-(1→4)]-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*	B	368
3-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose	B	369

(339) G. O. Aspinall and A. Nicholson, *J. Chem. Soc.*, 2503 (1960).
(340) P. K. Dhar and S. Mukherjee, *J. Sci. Ind. Research* (India), **18B**, 219 (1959).
(341) J. Ashby, J. Brooks, and W. W. Reid, *Chem. & Ind.* (London), 360 (1955).
(342) H. J. Phaff and B. S. Luh, *Arch. Biochem. Biophys.*, **36**, 231 (1952).
(343) A. L. Demain and H. J. Phaff, *Arch. Biochem. Biophys.*, **51**, 114 (1954).
(344) R. L. Whistler and H. E. Conrad, *J. Am. Chem. Soc.*, **76**, 3544 (1954).
(345) G. O. Aspinall, E. L. Hirst, and N. K. Matheson, *J. Chem. Soc.*, 989 (1956).
(346) F. Hostettler and H. Deuel, *Helv. Chim. Acta*, **34**, 2440 (1951).
(347) G. P. Mathur and S. Mukherjee, *J. Sci. Ind. Research* (India), **13B**, 452 (1954).
(348) A. J. Charlson, J. R. Nunn, and A. M. Stephen, *J. Chem. Soc.*, 1428 (1955).
(349) R. D. Hotchkiss and W. F. Goebel, *J. Biol. Chem.*, **115**, 285 (1936).
(350) A. J. Roudier and L. Eberhard, *Bull. soc. chim. France*, **28**, 2074 (1960).
(351) B. Lindberg and L. Sellerby, *Acta Chem. Scand.*, **14**, 1051 (1960).
(352) E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 1174 (1938).
(353) E. L. Hirst, E. E. Percival, and R. S. Williams, *J. Chem. Soc.*, 1942 (1958).
(354) J. J. O'Donnell and E. E. Percival, *J. Chem. Soc.*, 2168 (1959).
(355) J. K. Hamilton, D. Spriestersbach, and F. Smith, *J. Am. Chem. Soc.*, **79**, 443 (1957).
(356) W. D. S. Bowering and T. E. Timell, *J. Am. Chem. Soc.*, **82**, 2827 (1960).
(357) C. T. Bishop, *Can. J. Chem.*, **33**, 1521 (1955).
(358) C. T. Bishop, *Can. J. Chem.*, **31**, 134 (1953).
(359) R. L. Whistler and L. Hough, *J. Am. Chem. Soc.*, **75**, 4919 (1953).
(360) R. L. Whistler and D. I. McGilvray, *J. Am. Chem. Soc.*, **77**, 2212 (1955).
(361) D. L. Vincent, *Chem. & Ind.* (London), 1109 (1960).
(362) G. Jayme and K. Kringstad, *Chem. Ber.*, **93**, 2263 (1960).

(363) P. C. Das Gupta and P. B. Sarkar, *Textile Research J.*, **24**, 705 (1954).
(364) P. Andrews and J. K. N. Jones, *J. Chem. Soc.*, 1724 (1954).
(365) J. K. N. Jones and J. R. Nunn, *J. Chem. Soc.*, 3001 (1955).
(366) A. Roudier and L. Eberhard, *Compt. rend.*, **247**, 1505 (1958).
(367) G. A. Adams, *Can. J. Chem.*, **37**, 29 (1959).
(368) T. E. Timell, *Chem. & Ind.* (London), 999 (1959).
(369) D. J. Brasch and L. E. Wise, *Tappi*, **39**, 581, 768 (1956).

A16

[*Reprinted from the Proceedings of the Biochemical Society*, 14–15 *May* 1964.
Biochem. J., 1964, Vol. 92, No. 1, 20P.]

α-Galactosidase and Alkaline β-Fructofuranosidase Activity in *Vicia faba* Seeds

By J. B. PRIDHAM and MARY W. WALTER. (*Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey*)

Dormant *V. faba* seeds contain an α-galactosidase (α-D-galactoside galactohydrolase) which exhibits a maximum activity at pH 4·8 using melibiose as a substrate. Thiol groups on the enzyme are essential for its activity and the rates of hydrolysis for some galactose-containing oligosaccharides are in the following order: melibiose > raffinose > stachyose. Incubation of the enzyme preparation with melibiose and sucrose yields raffinose and a small amount of planteose as transfer products. Stachyose is formed when raffinose alone is used as substrate. In many respects the enzyme is similar to the α-galactosidase present in coffee beans (Anagnostopoulos, Courtois & Petek, 1955; Courtois, Wickström & le Dizet, 1956). It is doubtful whether these enzymes have any synthetic function *in vivo*; the raffinose family of oligosaccharides are probably derived from uridine diphosphate galactose and sucrose (Bourne, Pridham & Walter, 1962; Rast, McInnes & Neish, 1963).

Extracts of immature and germinated *V. faba* seeds exhibit a normal β-fructofuranosidase (β-D-fructofuranoside fructohydrolase) activity, the optimum pH for sucrose hydrolysis being 5·1. This type of activity is, however, absent in the dormant seeds which contain, instead, an alkaline β-fructofuranosidase with a pH optimum of 7·8. This enzyme liberates fructose from sucrose, raffinose and stachyose and can also transfer a β-fructofuranosyl residue to sucrose with the formation of 1^F-β-fructosylsucrose. The specifity and transferase activity of this enzyme is different from that of the alkaline sucrase obtained by Cooper & Greenshields (1961) from *Phaseolus vulgaris* seeds but is somewhat similar to the neutral β-fructofuranosidase found in *Saccharum* sp. (Hatch & Glaziou, 1963).

We are indebted to Professor E. J. Bourne and Dr R. N. Greenshields for helpful discussions. One of us (M.W.W.) received financial support from the Agricultural Research Council.

Anagnostopoulos, C., Courtois, J. E. & Petek, F. (1955). *Arch. Sci. biol., Napoli*, **39**, 631.
Bourne, E. J., Pridham, J. B. & Walter, M. W. (1962). *Biochem. J.* **82**, 44P.
Cooper, R. A. & Greenshields, R. N. (1961). *Nature, Lond.*, **191**, 601.
Courtois, J. E., Wickström, A. & le Dizet, P. (1956). *Bull. Soc. chim. biol.* **38**, 851.
Hatch, M. D. & Glaziou, K. T. (1963). *Plant. Physiol.* **38**, 344.
Rast, D., McInnes, A. G. & Neish, A. C. (1963). *Canad. J. Bot.* **41**, 1681.

Biochem. J. (1965) **97**, 802
Printed in Great Britain

The Biosynthesis of Raffinose

BY E. J. BOURNE, MARY W. WALTER AND J. B. PRIDHAM
Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 14 *June* 1965)

1. Reaction of UTP and α-D-galactose 1-phosphate with [U-^{14}C]sucrose in the presence of a *Vicia faba* dormant-seed preparation yielded the trisaccharide raffinose. 2. UTP–α-D-galactose 1-phosphate-uridylyltransferase activity has been demonstrated in the bean preparation and evidence for the participation of UDP-galactose in the trisaccharide synthesis is presented. 3. UDP-galactose 4-epimerase is present in the dormant seed. 4. The biosynthesis of raffinose in relation to the metabolism of other carbohydrates in plants is discussed.

During the past 15 years it has become apparent that much of the carbohydrate metabolism in both plants and animals involves nucleoside diphosphate sugar derivatives (e.g. Feingold, Neufeld & Hassid, 1964; Ginsburg, 1964).

Sucrose is the major oligosaccharide found in plants and the formation of this sugar by the transfer of D-glucose from UDP-glucose to D-fructose has been demonstrated with transferases from several different species (e.g. Turner, 1954; Cardini, Leloir & Chiriboga, 1955; Bean & Hassid, 1955; Burma & Mortimer, 1956; Rorem, Walker & McCready, 1960). In addition, Leloir & Cardini (1955) showed that a wheat-germ preparation catalysed the formation of sucrose phosphate from UDP-glucose and D-fructose 6-phosphate. Trehalose, the non-reducing disaccharide found in yeasts and fungi, appears to be formed by the action of a specific phosphatase on trehalose 6-phosphate, the latter being derived from UDP-glucose and D-glucose 6-phosphate (Leloir & Cabib, 1953; Cabib & Leloir, 1958). After sucrose, the next most abundant oligosaccharide found in plant tissues is raffinose[α-D-Gal$_p$-(1→6)-β-D-Glc$_p$-(1→2)-β-D-Fru$_f$] (French, 1954; Courtois, 1959). The biosynthesis of this compound has already been achieved *in vitro* by allowing sucrose to react with α-D-galactopyranosides, such as phenyl α-D-galactoside and melibiose, in the presence of α-D-galactoside galactohydrolase (e.g. Courtois, Petek & To Dong, 1961; Pridham & Walter, 1964). This type of simple transferase reaction is, however, of doubtful biological importance and it is perhaps more reasonable to assume that *in vivo* the D-galactopyranosyl residue in raffinose is derived from a nucleoside diphosphate D-galactose derivative. The investigation described in this paper strongly supports this suggested mechanism. A preliminary account of the results has already been published (Bourne, Pridham & Walter, 1962).

METHODS

General methods. Absorption spectra were measured in alkaline solution with a Perkin–Elmer model 137 u.v. spectrophotometer (1cm. cell). All solutions were concentrated with a rotatory evaporator at temperatures not exceeding 40°.

Paper chromatography. Whatman no. 1 paper and the following solvent systems were used for the examination of carbohydrates: *A*, ethyl acetate–acetic acid–water (9:2:2, by vol.); *B*, ethyl acetate–pyridine–water (2:1:2, by vol.). *p*-Anisidine hydrochloride (spray *a*) (Hough, Jones & Wadman, 1950) was used to detect reducing sugars and galactosylsucrose derivatives. Silver nitrate–NaOH (spray *b*) (Trevelyan, Procter & Harrison, 1950) was used to locate reduced mono- and di-saccharides. Fructose-containing compounds were detected with urea–phosphate reagent (spray *c*) (Wise, Dinbew & Davies, 1955). Nucleotides were chromatographed with ethanol–M-ammonium acetate (7:3, v/v, pH 7·5) (solvent *C*) and located by u.v. absorption by using a Chromatolite lamp (Hanovia Ltd., Slough, Bucks.).

Paper electrophoresis. Carbohydrates were examined by this procedure with Whatman no. 3 paper and 0·2M-sodium borate, pH 10·0, and 8mM-ammonium molybdate, pH 5·2, solutions as electrolytes. Before treatment with spray *a*, borate electrophoretograms were exposed to HCl fumes for 10min. Whatman no. 1 paper with 0·1M-ammonium formate buffer, pH 3·7, was used for the electrophoresis of nucleotides.

Densitometer method for determination of sugars. Known volumes of standard solutions of D-galactose, sucrose, raffinose and stachyose were spotted on to Whatman no. 1 papers, which were developed with solvent *A*. The papers were then treated with spray *a* and heated at 120° for 5min. The chromatograms were cut into strips and the coloured spots measured with an EEL absorption densitometer (Evans Electroselenium Ltd., Halstead, Essex). A comparison of the areas under the absorption curves with the sugar concentrations showed that there were linear relation-

ships with all four compounds and that the errors did not exceed $\pm 7\%$ over the range 20–90 μg.

Examination of sugars in leguminous seeds. The seeds were removed from the pods in July and extracted with cold aq. 75% (v/v) methanol. The extracts, after concentration, were examined on paper chromatograms with solvent *A* (sprays *a* and *c*). Other seed samples from the same species were left for 10 days in the air at room temperature before examination.

Ten samples of green *Vicia faba* seeds (ten beans/sample) were weighed and placed in a laboratory cupboard in an open Petri dish. At 24hr. intervals samples were reweighed and extracted with aq. 75% (v/v) methanol (25ml.). The extracts were concentrated to 5ml., and samples were taken with a micrometer syringe and spotted on to Whatman no. 1 papers. These were developed with solvent *A* and the amounts of D-glucose, sucrose, raffinose and higher galactosylsucrose derivatives (the latter expressed as μg. of stachyose) present were determined by the densitometer method.

Enzyme preparation. The testas were removed from dormant *V. faba* (var. Johnson's long pod) seeds and the cotyledons and embryos powdered at room temperature in a vibratory ball mill. Extraction of the powder was achieved by maceration in a homogenizer (Measuring and Scientific Equipment Ltd., London) with 0·5M-sodium phosphate buffer, pH7·0 (1·5ml. of buffer/g. of powder), for 1min. at 0–5°. The gross tissue residue was removed by centrifugation at 1600*g* for 30min. at 0° and the supernatant dialysed against 20vol. of phosphate buffer at 5°.

Synthesis of raffinose. The enzyme (0·3ml.) was incubated with ATP (10 μmoles), UTP (3 μmoles), α-D-galactose 1-phosphate (5 μmoles), [U-^{14}C]sucrose (4 μmoles; 20 μc), $MgCl_2$ (10 μmoles) and L-cysteine (3 μmoles) at 35°. Identical reaction mixtures, but (*a*) with boiled enzyme and (*b*) with UTP and ATP omitted, were also set up. After 2·5hr., ethanol (0·3ml.) was added to each reaction mixture and the solutions were streaked on to Whatman no. 3 papers. These were developed with solvent *A* for 24hr. and radioautographs prepared (Ilford industrial G X-ray film; 4 days exposure). In the reaction mixture that contained boiled enzyme the [U-^{14}C]sucrose appeared to be unchanged, and the one containing no exogenous nucleotides showed labelled bands corresponding to sucrose, glucose, fructose and a trisaccharide (*K*; R_{Glc} 0·25; see Pridham & Walter, 1964). The complete reaction mixture exhibited a similar chromatographic pattern but an additional labelled compound (*R*) with R_F identical with that of raffinose was also observed. A scan of the radioactivity on the chromatogram of the latter reaction mixture is shown in Fig. 1. This was constructed by passing a 1cm. strip of the paper under an aluminium plate possessing a 0·5cm. × 1·0cm. slit and measuring the radiation with a Geiger–Müller tube (2mg./cm.^{2} window) connected to a conventional scaler.

Isolation and examination of R. The band containing *R* was eluted with water and the solution concentrated to a syrup. Unlabelled raffinose (4mg.) was added and the whole (*R'*) dissolved in water (1·5ml.). Appropriate volumes of this solution were then subjected to further paper-chromatographic (solvents *A* and *B*, spray *a*) and electrophoretic (borate buffer, spray *a*) analysis. [Paper chromatograms and electrophoretograms were in all cases scanned for radioactivity, as described above, and then the coloured spots resulting from treatment with the carbohydrate-locating reagent examined with an absorbance densitometer (see Fig. 2). This procedure was also used to investigate all the products of the degradative reactions described below.]

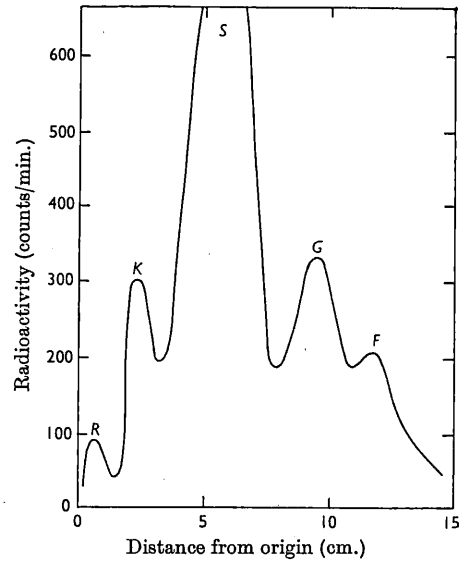


Fig. 1. Scan of the ^{14}C-labelled compounds present after paper-chromatographic fractionation of the raffinose-synthesizing system described in the text. *R*, Raffinose; *K*, 1-kestose; *S*, sucrose; *G*, glucose; *F*, fructose.

Acid hydrolysis of R'. The solution of *R'* (0·5ml.) was heated with N-H_2SO_4 (3ml.) at 100° for 3hr. After neutralization with Amberlite IR-4B (OH^- form) resin the hydrolysate was examined on a paper chromatogram (solvent *A*, spray *a*).

Treatment of R' *with α- and β-D-galactoside galactohydrolases.* The raffinose-synthesizing enzyme from dormant *V. faba* seeds was used as a source of α-galactosidase (see Pridham & Walter, 1964), and an *Escherichia coli* β-galactosidase preparation was kindly provided by Professor K. Wallenfels. The enzymes (0·1ml. volumes) were incubated with solutions of *R'* (0·1ml.) at 35° for 12hr. and the mixtures examined on paper chromatograms (solvent *A*, spray *a*).

Hydrolysis of R' *with β-D-fructofuranoside fructohydrolase.* Yeast invertase concentrate (British Drug Houses Ltd.; diluted 10-fold with 0·2M-sodium acetate buffer, pH4·8; total volume 0·1ml.) was incubated with *R'* (0·1ml.) at 35° for 3hr. The reaction mixture was examined on paper chromatograms (solvent *A*, spray *a*) and electrophoretograms (borate buffer, spray *a*).

Reduction of melibiose. The solution remaining after the invertase hydrolysis was diluted to 1ml. with water and KBH_4 (2mg.) added. After 4hr. the reaction mixture was deionized with Bio-Deminrolit (HCO_3^- form) resin and the radioactive product (melibi-itol) isolated by paper electrophoresis (molybdate buffer; side strip treated with spray *b*) followed by elution of the compound from the paper with water.

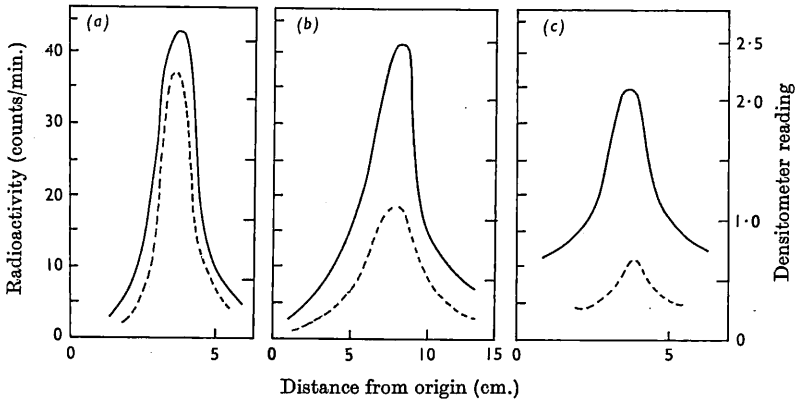


Fig. 2. (*a*) Paper chromatography with solvent *A*, (*b*) paper chromatography with solvent *B* and (*c*) paper electrophoresis in borate buffer of raffinose (*R'*). Details are given in the text. Continuous lines represent the radioactivity and broken lines the colour intensity of the spots produced by reaction with *p*-anisidine hydrochloride.

Hydrolysis of melibi-itol. The syrup remaining after concentration of the above eluate was heated with N-H_2SO_4 (0·5ml.) at 100° for 3hr. Electrophoresis of the hydrolysate (borate buffer, spray *b*) after neutralization with Amberlite IR-4B (OH^- form) resin revealed a radioactive spot with the same mobility as D-glucitol.

Biosynthesis of UDP-galactose. UTP (0·8μmole), α-D-galactose 1-phosphate (1·5μmoles), ATP (0·2μmole), $MgCl_2$ (1μmole) and L-cysteine (0·3μmole) were incubated with the dormant-bean preparation (0·1ml.) for 1·5hr. at 35°. The reaction mixture was then examined on a paper chromatogram (solvent *C*) and a u.v.-absorbing spot that co-chromatographed with UDP-galactose was eluted from the paper with water. The spectrum of the resulting solution was measured and a sample also subjected to paper electrophoresis (formate buffer). Hydrolysis of the nucleotide was effected by heating the aqueous solution with Amberlite IR-120 (H^+ form) resin at 100° (5min.). The liberated sugar (galactose) was identified by paper chromatography (solvent *A*, spray *a*). A control reaction from which UTP had been omitted yielded no u.v.-absorbing band in the UDP-hexose region of the paper chromatogram.

The u.v.-absorbing band, corresponding to UDP-galactose on a paper chromatogram (solvent *C*), that resulted from the incubation (1hr. at 35°) of UDP-glucose (0·3μmole), NAD^+ (0·15μmole), ATP (0·2μmole), $MgCl_2$ (1·0μmole) and L-cysteine (0·3μmole) with the dormant-bean preparation (0·1ml.) was also examined by the same methods.

Attempted biosynthesis of ADP-galactose. The bean preparation (0·1ml.) was incubated for 2·5hr. at 35° with ATP (0·6μmole), α-D-galactose 1-phosphate (2·2μmoles), $MgCl_2$ (1μmole) and L-cysteine (0·3μmole). The reaction mixture was examined on a paper chromatogram (solvent *C*).

RESULTS AND DISCUSSION

Raffinose, stachyose and higher-molecular-weight α-D-galactosylsucrose derivatives are common constituents of the seeds of legumes (French, 1954; Courtois, 1959), where they appear to be formed during the final stages of maturation (e.g. Korytnyk & Metzler, 1962; Gould & Greenshields, 1964). A qualitative chromatographic comparison of the sugars in the seeds of *Colutea arborescens*, *Cytissus scoparius*, *Selega orientalis* and *Lupinus polyphyllus* at an immature stage and after dehydration at room temperature confirmed this. A quantitative illustration of the changes in sugar concentrations in green *V. faba* seeds that were taken from the pod and left in the air for several days is given in Fig. 3. This shows that the loss of water by the tissues is roughly paralleled by an increase in raffinose and higher homologues. Sucrose exhibited an initial rapid decrease in concentration followed by an increase after 3 days, and the glucose content of the tissues slowly declined. It should, however, be emphasized that Fig. 3 may not be an exact illustration of the changes that occur *in vivo* as the 'maturation' was probably accelerated by removal of the bean seeds from the pods, thus increasing the rate of dehydration.

Incubation of an extract of mature broad-bean seeds with [U-^{14}C]sucrose, α-D-galactose 1-phosphate, UTP and ATP produced a labelled compound *R* (see Fig. 1) with chromatographic properties characteristic of raffinose together with a second radioactive trisaccharide *K*. When the nucleoside triphosphates were omitted from the reaction mixture *K* was still produced but not *R*. *K* has been identified as 1^F-β-D-fructosylsucrose (1-kestose) (Pridham & Walter, 1964) and is presumably formed by the action of an 'alkaline' β-D-fructofuranoside fructohydrolase on sucrose.

Compound *R* was mixed with unlabelled raffinose (mixture *R'*) and further examined on paper

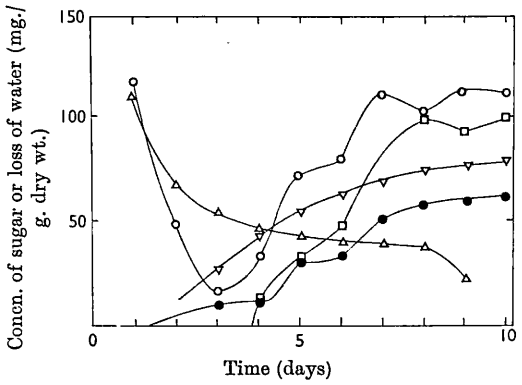


Fig. 3. Changes in mono- and oligo-saccharide content during dehydration of *V. faba* seeds. Details are given in the text. ○, Sucrose; □, stachyose; ▽, loss of water; ●, raffinose; △, glucose.

chromatograms (two solvent systems) and by paper electrophoresis (borate buffer). By scanning the papers for radioactivity and by using a densitometer to examine the coloured spots produced by reaction with *p*-anisidine hydrochloride, an exact coincidence between colour and radioactivity was observed in all cases (Fig. 2). This procedure was also used to examine the products resulting from acidic and enzymic degradation of the mixture *R'*. In this way it was shown that complete acid hydrolysis yielded galactose and labelled glucose and fructose and that treatment with a *V. faba* α-D-galactoside galactohydrolase preparation produced galactose and [^{14}C]sucrose. No hydrolysis was observed on incubation of *R'* with *E. coli* β-D-galactoside galactohydrolase. A labelled disaccharide that was indistinguishable from melibiose on paper chromatograms and electrophoretograms was formed when yeast β-D-fructofuranoside fructohydrolase was used as the hydrolytic agent. The identity of this disaccharide was further checked by reduction with potassium borohydride followed by electrophoresis in ammonium molybdate solution. The resulting non-reducing derivative was indistinguishable from authentic melibi-itol on electrophoresis. The rate of movement of melibi-itol in this electrolyte (M_{glucitol} 0·8) is characteristic of a 6-substituted hexitol derivative (Bourne, Hutson & Weigel, 1959). Acid hydrolysis of the melibi-itol yielded labelled glucitol. This degradative study therefore confirmed that *R* was raffinose and that it was formed by the transfer of D-galactose to ^{14}C-labelled sucrose.

Incubation of α-D-galactose 1-phosphate with UTP and the bean enzyme preparation produced a compound that behaved as a UDP-hexose derivative on paper chromatograms and electrophoretograms and had a u.v.-absorption spectrum characteristic of a uridine nucleotide (E_{250}/E_{260}, 0·8; E_{280}/E_{260}, 0·24). Acid hydrolysis of this compound liberated galactose. It appeared therefore that the bean preparation possessed UTP–α-D-galactose 1-phosphate-uridylyltransferase activity (cf. Neufeld, Ginsburg, Putman, Fanshier & Hassid, 1957) and hence that UDP-galactose served as the galactose donor for raffinose synthesis. Passeron, Recondo & Dankert (1964) have, however, demonstrated the presence of ATP–α-D-galactose 1-phosphate-adenylyltransferase activity in wheat germ, and it was conceivable that ADP-galactose was formed and acted as a galactose donor in the raffinose-synthesizing system. All attempts in the present work to synthesize ADP-galactose from α-D-galactose 1-phosphate and ATP in the presence of the bean enzyme failed, and it is therefore unlikely that ADP-galactose played any significant role in the synthesis of the trisaccharide. Further evidence of the participation of UDP-galactose in raffinose formation has also been obtained by allowing UDP-[1-^{14}C]galactose to react with sucrose and the bean preparation. This resulted in approx. 39% incorporation of [1-^{14}C]galactose after 2·5hr. incubation at 37° (Pridham & Hassid, 1965).

It is probable that *in vivo* the same pathway is responsible for the synthesis of raffinose and possibly other α-D-galactosylsucrose derivatives. Rast, McInnes & Neish (1963) have come to the same conclusion from a study of ^{14}C-incorporation into the carbohydrates of spruce twigs.

UDP-galactose is, no doubt, widespread in plant tissues (e.g. Ginsburg, Stumpf & Hassid, 1956; Brown, 1962) and has actually been isolated from dormant and germinating *V. faba* seeds (J. B. Pridham & M. W. Walter, unpublished work). UDP-galactose can also be readily derived from UDP-glucose by the action of UDP-galactose 4-epimerase (cf. Neufeld *et al.* 1957) and this enzyme has now been shown to be present in bean seeds. Thus the incubation of UDP-glucose with NAD^+ and the bean enzyme preparation yielded a nucleotide that was identified as UDP-galactose by the same procedures described above for the examination of the product of UTP–α-D-galactose 1-phosphate-uridylyltransferase activity. A pathway from UDP-glucose to raffinose via UDP-galactose is therefore very likely to exist in *V. faba* (and other seeds), as all the necessary enzymes and their substrates are present in the tissues. Dupéron (1964) has observed that ^{14}C-labelled glucose rapidly gives rise to labelled sucrose and raffinose in *Phaseolus vulgaris* seeds. The nucleotide-dependent pathway would also allow raffinose formation to be closely linked to starch and sucrose metabolism.

The possible role of α-D-galactoside galactohydrolase in the formation of raffinose and its homologues should perhaps not be entirely dismissed at the present time. Pridham & Walter (1964) have shown that this enzyme from *V. faba* seeds will transfer galactose from melibiose to sucrose *in vitro* with the formation of raffinose and small amounts of planteose [α-D-Glc_p-(1→2)-β-D-Fru_f-(6← 1)-α-D-Gal_p; it may be significant that this latter sugar does not appear to be present in *V. faba*] and that raffinose in the presence of this enzyme can act as both galactose donor and acceptor with the consequent formation of stachyose [α-D-Gal_p-(1→6)-α-D-Gal_p-(1→6)-α-D-Glc_p-(1→2)-β-D-Fru_f] (cf. Courtois *et al.* 1961). If such a pathway is responsible for the formation of raffinose *in vivo* then the galactose donor for the reaction will have to be established. No naturally occurring low-molecular-weight compounds that would be suitable substrates have so far been identified, although the possible existence of galactose donors of high degree of polymerization should be borne in mind. For example, Courtois & Pecheron (1961) have shown that an α-D-galactoside galactohydrolase from *Trigonellum foenum graecum* seeds can synthesize raffinose by transfer of galactose from a galactomannan to sucrose. The equilibrium constants for glycohydrolase-catalysed reactions normally favour hydrolysis rather than the synthesis of glycosidic bonds. Hence synthesis is normally forced by the use of high (non-physiological) substrate concentrations and this has led to scepticism about the part played by these enzymes in biosynthesis. There is, however, little information on the effective concentrations of potential substrates in the plant cell. In addition, most of the studies *in vitro* have been carried out with very crude enzymes. Clearly the role of α-D-galactoside galactohydrolase and other glycohydrolases in synthetic processes in the plant require further investigation but, on the basis of existing evidence, the nucleotide-dependent pathways for the formation of the principal oligosaccharides occurring in higher plants appear to be the more important. With sucrose there is virtually no doubt about the nature of the reaction; the enzymic synthesis of this disaccharide has never been achieved with higher-plant enzymes in the absence of a nucleoside diphosphate derivative of D-glucose.

M. W. W. is indebted to the Agricultural Research Council for a Studentship.

REFERENCES

Bean, R. C. & Hassid, W. Z. (1955). *J. Amer. chem. Soc.* **77**, 5737.

Bourne, E. J., Hutson, D. H. & Weigel, H. (1959). *Chem. & Ind.* p. 1047.

Bourne, E. J., Pridham, J. B. & Walter, M. W. (1962). *Biochem. J.* **82**, 44P.

Brown, E. G. (1962). *Biochem. J.* **85**, 633.

Burma, D. P. & Mortimer, D. C. (1956). *Arch. Biochem. Biophys.* **62**, 16.

Cabib, E. & Leloir, L. F. (1958). *J. biol. Chem.* **231**, 259.

Cardini, C. E., Leloir, L. F. & Chiriboga, J. (1955). *J. biol. Chem.* **214**, 149.

Courtois, J. E. (1959). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 1, p. 140.

Courtois, J. E. & Pecheron, F. (1961). *Bull. Soc. Chim. biol., Paris*, **43**, 167.

Courtois, J. E., Petek, F. & To Dong (1961). *Bull. Soc. Chim. biol., Paris*, **43**, 1189.

Dupéron, R. (1964). *C.R. Acad. Sci., Paris*, **258**, 5960.

Feingold, D. S., Neufeld, E. F. & Hassid, W. Z. (1964). In *Modern Methods of Plant Analysis*, vol. 7, p. 474. Ed. by Linskens, H. F., Sanwal, B. D. & Tracey, M. V. Berlin: Springer-Verlag.

French, D. (1954). *Advanc. Carbohyd. Chem.* **9**, 149.

Ginsburg, V. (1964). *Advanc. Enzymol.* **26**, 35.

Ginsburg, V., Stumpf, P. K. & Hassid, W. Z. (1956). *J. biol. Chem.* **223**, 977.

Gould, M. F. & Greenshields, R. N. (1964). *Nature, Lond.*, **202**, 108.

Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.

Korytnyk, W. & Metzler, E. (1962). *Nature, Lond.*, **195**, 616.

Leloir, L. F. & Cabib, E. (1953). *J. Amer. chem. Soc.* **75**, 5445.

Leloir, L. F. & Cardini, C. E. (1955). *J. biol. Chem.* **214**, 157.

Neufeld, E. F., Ginsburg, V., Putman, E. W., Fanshier, D. & Hassid, W. Z. (1957). *Arch. Biochem. Biophys.* **69**, 602.

Passeron, S., Recondo, E. & Dankert, M. (1964). *Biochim. biophys. Acta*, **89**, 372.

Pridham, J. B. & Hassid, W. Z. (1965). *Plant Physiol.* (in the Press).

Pridham, J. B. & Walter, M. W. (1964). *Biochem. J.* **92**, 20P.

Rast, D., McInnes, A. G. & Neish, A. C. (1963). *Canad. J. Bot.* **41**, 1681.

Rorem, E. S., Walker, H. G. & McCready, R. M. (1960). *Plant Physiol.* **35**, 269.

Trevelyan, W. E., Procter, D. P. & Harrison, J. B. (1950). *Nature, Lond.*, **166**, 444.

Turner, J. F. (1954). *Nature, Lond.*, **174**, 692.

Wise, C. S., Dinbew, R. J. & Davies, H. A. (1955). *Analyt. Chem.* **27**, 33.

Biosynthesis of Raffinose[1, 2]

By

J. B. Pridham and W. Z. Hassid

Department of Biochemistry, University of California, Berkeley, California

Reprinted from PLANT PHYSIOLOGY, Vol. 40, No. 6, November, 1965, pages 984–986.

(PRINTED IN U.S.A.)

Biosynthesis of Raffinose[1, 2]

J. B. Pridham and W. Z. Hassid
Department of Biochemistry, University of California, Berkeley, California

Sucrose, the ubiquitous constituent of higher plant tissues, is frequently accompanied by higher molecular weight oligosaccharides containing D-galactose residues. The commonest of these are raffinose (α-D-gal$_p$-(1 → 6)-α-D-G$_p$-(1 → 2)-β-D-Fru$_f$) and higher homologues which occur in relatively high concentrations in many dormant seeds and other plant organs (3, 6). Raffinose and stachyose (α-D-Gal$_p$-(1 → 6)-α-D-Gal$_p$-(1 → 6)-α-D-G$_p$-(1 → 2)-β-D-Fru$_f$) have also been detected in sieve-tube exudates from several species and, therefore, presumably form part of the organic material which is translocated (10).

The biosynthesis of the raffinose family of oligosaccharides occurs during seed maturation and in vitro it has been demonstrated that raffinose is formed when sucrose,α-D-galactose 1-phosphate and UTP[3] are incubated with an enzyme preparation from dormant broad bean (*Vicia faba*) seeds (1). This paper describes further studies which implicate UDP-D-galactose as the galactose donor in this system.

Materials and Methods

UDP-D-Galactose-1-C^{14} (7.2) (μc/μM). This was prepared according to the method of Watkins and Hassid (9).

Paper Chromatography. Separations were effected on Whatman No. 1 paper using ethyl acetate-acetic acid-water (9:2:2, v/v) solvent. *p*-Anisidine phosphate reagent was used to locate sugars (5).

Paper Electrophoresis. Whatman No. 1 paper was used with 0.2 M ammonium formate (pH 3.6) or 0.05 M sodium borate (pH 9.0) buffers at 30 v/cm.

Measurement of Radioactivity. Paper chromatograms and electrophoretograms were scanned with a Nuclear Chicago Actigraph II coupled to an analytical count-rate meter. Papers were also cut into strips and counted directly with a Geiger-Müller tube and rate-meter and/or the strips were eluted with water and the eluates taken to dryness and counted on planchets.

Enzyme Preparation. The testas were removed from dormant broad-beans (*Vicia faba* var. Imperial Long pod) and the seeds powdered (40 mesh) in a Wiley mill. The powder (44.6 g) was then stirred with 0.1 M sodium phosphate buffer (pH 7.0; 60 ml) at 4° for 3 hours. After filtering through cheesecloth, the extract was centrifuged at 7700 × *g* for 2 minutes at 0° and the supernatant then dialyzed against 0.05 M sodium phosphate buffer (pH 7.0; 2 × 2 liters) for 48 hours at 4°

Raffinose Formation. The reactions were carried out in glass capillary tubes (7) and each mixture contained sucrose (0.5 μM), UDPgal-1-^{14}C (0.02 μc; 7.2 μc/μM), ATP (0.5 μM), enzyme (10 μl) and 0.5 M sodium phosphate buffer (pH 7.0; 2 μl) in a total volume of 24 μl. Incubations were carried out at 37°.

In one experiment (A) the reaction mixtures were spotted on to a paper chromatogram after incubation for 1 and 2.5 hours. After development for 18 hours, the paper was dried and scanned for radioactivity (see fig 1). The regions corresponding to raffinose on the chromatogram were eluted with water, combined and concentrated under reduced pressure at room temperature. The resulting solution was then examined on a paper electrophoretogram (sodium borate buffer).

In a second experiment (B) a 2-hour reaction mixture was subjected to paper electrophoresis (ammonium formate buffer) and the lower three-fourths of the paper then carefully washed with water, to remove nucleotides and sugar phosphates, and dried. Raffinose, sucrose, and D-galactose standards were applied to the origin of the electrophoretogram (allowing for electroendosmosis) and the paper was then developed with the ethyl acetate-acetic acid-water solvent (18 hours) and scanned for radioactivity (fig 2). The band corresponding to raffinose was eluted and concentrated to approximately 30 μl and authentic raffinose (3.6 mg) added. Aliquots (10 μl) were subjected to: 1) further paper chromatographic examination; 2) hydrolysis with 2 N HCl (10 μl) at 100° for 2.5 hours; 3) hydrolysis with yeast invertase concentrate (4 μl) at 37° for 4.5 hours. The 2 hydrolysates were then resolved on paper chromatograms and the papers sprayed carefully with *p*-anisidine phosphate reagent and finally examined for radioactivity.

---

[1] Received May 11, 1965.
[2] This investigation was supported in part by research grants from the National Institutes of Health, United States Public Health Service (No. A-1418) and from the National Science Foundation (No. G-23763). Support of this work by the Agricultural Experiment Station is also acknowledged.
[3] Abbreviations; ATP, adenosine 5'-triphosphate; UDP-D-galactactose, Uridine 5'-(D-galactosylpyrophosphate); UTP, Uridine 5'-triphosphate.

Results

An examination of the radioactive products from experiment A (fig 1) indicated the presence of UDP-D-galactose, a disaccharide (which has not been further investigated) and a compound which co-chromatographed and had the same mobility on co-electrophoresis with authentic raffinose. The amount of label incorporated into the raffinose was 33 and 39 % after 1 and 2.5 hours, respectively.

In the second experiment (B) more labeled trisaccharide (fig 2) was obtained and this was mixed with authentic raffinose. Further chromatography of this mixture confirmed that the labeled trisaccharide was raffinose. In addition, hydrolysis of the mixture with 1 N HCl yielded labeled galactose and hydrolysis with invertase (β-D-fructofuranoside fructohydrolase; 3.2.1.25) produced a radioactive disaccharide which was indistinguishable from melibiose on a paper chromatogram.

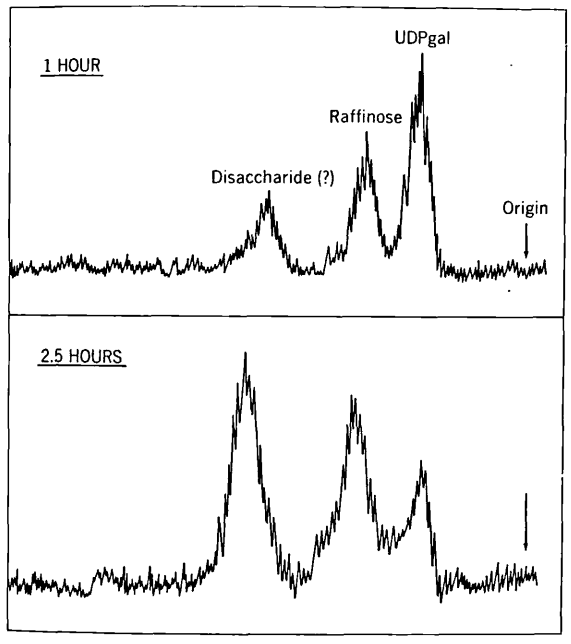


FIG. 1. Strip-counter tracing of products (separated by chromatography) formed in raffinose synthesizing system (Expt A).

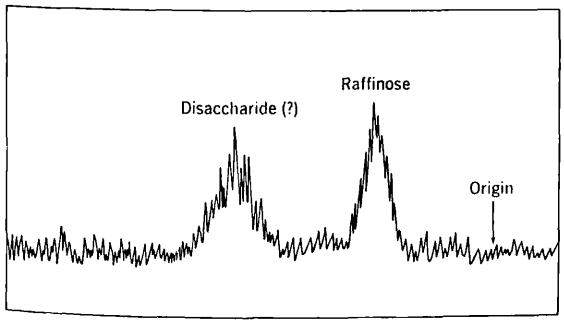


FIG 2. Strip-counter tracing of products (separated on a paper chromatogram) remaining after removal of nucleotides and sugar phosphates from raffinose synthesizing system (Expt. B).

Discussion

The results described in this paper, particularly when considered in conjunction with earlier observations (1), show clearly that *Vicia faba* seeds contain an enzyme which can synthesize raffinose in vitro by the enzymic transfer of D-galactose from UDP-D-galactose to sucrose. Although the enzyme preparation was crude, the percentage incorporation of label was relatively high and it is, therefore, probable that this mechanism functions in maturing seeds. The involvement of UDP-D-galactose in the reaction would be expected, since by analogy with sucrose synthetase action (2) the equilibrium constant should favor synthesis and, in addition, raffinose formation would be closely linked, by known reactions, to the general carbohydrate metabolism of the tissues. It is possible that other nucleoside diphosphate galactose derivatives are involved and this obviously requires further investigation.

The additional possibility that raffinose is formed by transfer of D-galactose from low energy donors to sucrose should not at the present time be completely discounted. The synthesis of raffinose and stachyose using such donors in the presence of α-galactosidase (α-D-galactoside galactohydrolase) preparations has already been demonstrated [e.g., Courtois, Petek and To Dong (4); Pridham and Walter (8)] although no suitable galactose donors for this type of reaction appear to occur naturally in plant tissues. The equilibrium constants for glycosidase-catalyzed reactions also normally favor hydrolysis, but in vivo factors such as localized high substrate concentrations or rapid utilization of products may encourage oligosaccharide synthesis.

Summary

An enzyme preparation was obtained from broad bean (*Vicia faba*) seeds capable of transferring D-galactose from UDP-D-galactose to sucrose, forming the trisaccharide, raffinose.

Literature Cited

1. BOURNE, E. J., J. B. PRIDHAM, AND M. W. WALTER. 1962. The biosynthesis of galactosylsucrose derivatives. Biochem. J. 82: 44P.
2. CARDINI, C. E., L. F. LELOIR, AND J. CHIRIBOGA. 1955. The biosynthesis of sucrose. J. Biol. Chem. 214: 149–55.
3. COURTOIS, J. E. 1958. Les oligosaccharides non réducteurs contenant du galactose. Proc. 4th Intern. Cong. Biochem., Vienna 1: 140–59.

4. COURTOIS, J. E., F. PETEK, AND TO DONG. 1961. Synthèse de plantéose par action transferante de l'α-galactosidase des graines de plantago. Bull. Soc. Chim. Biol. 43: 1189–96.
5. FEINGOLD, D. S., E. F. NEUFELD, AND W. Z. HASSID. 1958. Synthesis of a β-1,3-linked glucose by extracts of *Phaseolus aureus* seedlings. J. Biol. Chem. 233: 783–88.
6. FRENCH, D. 1954. The raffinose family of oligosaccharides. Advan. Carbohydrate Chem. 9: 149–81.
7. PORTER, W. L. AND N. HOBAN. 1954. Ultramicrotechnique for enzymic hydrolysis of sugars prior to chromatogram analysis. Anal. Chem. 26: 1846–48.
8. PRIDHAM, J. B. AND M. W. WALTER. 1964. α-Galactosidase and alkaline β-fructofuranosidase activity in *Vicia faba* seeds. Biochem. J. 92: 20P.
9. WATKINS, W. M. AND W. Z. HASSID. 1962. The synthesis of lactose by particulate enzyme preparations from guinea pig and bovine mammary glands: J. Biol. Chem. 237: 1432–40.
10. ZIMMERMANN, M. H. 1957. Translocation of organic substances in trees. I. The nature of sugars in the sieve-tube extracts of trees. Plant Physiol. 32: 288–91.

A19

[*Reprinted from the Proceedings of the Biochemical Society*, 17 *June* 1966.
Biochem. J., 1966, Vol. 100, No. 2, 22P.]

Oligosaccharide Metabolism in Seedlings

By J. B. PRIDHAM, MARY W. WALTER and H. G. J. WORTH. (*Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey*)

Raffinose and related oligosaccharides commonly form carbohydrate reserves in higher plants. In seeds these reserves rapidly disappear upon germination (e.g. MacLeod, 1957; Pridham, 1958; Pazur, Shadaksharaswamy & Meidell, 1962; Shiroya, 1963; Gould & Rees, 1965) and no free galactose accumulates in the tissues. To account for this Pazur *et al.* (1962) have suggested that in soyabean, galactose is liberated from the oligosaccharides by α-D-galactoside galactohydrolase and then further metabolized thus:

$$\text{D-Galactose} \xrightarrow{\text{ATP}} \alpha\text{-D-galactose 1-phosphate} \xrightarrow{\text{UTP}} \text{UDP-galactose.}$$

Raffinose is probably formed *in vivo* from UDP-galactose and sucrose (Bourne, Walter & Pridham, 1965; Pridham & Hassid, 1965) and therefore a conceivable alternative mechanism for the catabolism of raffinose by a reversal of raffinose synthetase activity was investigated.

Galactose, fructose and UDP-hexose were produced when UDP (1 μmole) and raffinose (2·5 μmole) were incubated at pH 7·0 (37°) with a 30–40% saturated ammonium sulphate fraction from germinating *Vicia faba* embryos (25 μl.; total volume 40 μl.). Further examination of the UDP-hexose showed that it was UDP-glucose. UDP-glucose 4-epimerase activity in the bean enzyme was negligible and when the preparation was incubated with raffinose alone galactose and sucrose were produced. It was apparent, therefore, that in the presence of UDP the preparation degraded raffinose not by reversal of raffinose synthetase action but by the following two stage process:

$$\text{Raffinose} \rightarrow \text{Sucrose} \xrightarrow{\text{UDP}} \text{UDP-glucose} + \text{Fructose}$$
$$+$$
$$\text{Galactose}$$

Such a mechanism may exist *in vivo* but the inhibition of raffinose degradation in *V. faba* seeds supplied with water under anaerobic conditions (cf. MacLeod, 1957; Shiroya, 1963; Gould & Rees, unpublished results) still requires explanation.

The *V. faba* embryo preparation readily produced UDP-glucose when raffinose was replaced by sucrose in the above reaction mixture (cf. Cardini, Leloir & Chiriboga, 1955; De Fekete & Cardini, 1964; Avigad, 1964; Murata, Sugiyama, Minamikawa & Akazawa, 1966). The apparent K_m for sucrose was $1{\cdot}5 \times 10^{-2}$ M. Activity was inhibited by UMP and, to a lesser extent, UTP; strong inhibition was observed with D-glucose and D-fructose. Possible control mechanisms which could affect the rate of sucrose metabolism in seeds will be discussed.

We are indebted to the Science Research Council for financial support.

Avigad, G. (1964). *J. biol. Chem.* **239**, 3613.
Bourne, E. J., Walter, M. W. & Pridham, J. B. (1965). *Biochem. J.* **97**, 802.
Cardini, C. E., Leloir, L. F. & Chiriboga, J. (1955). *J. biol. Chem.* **214**, 149.
De Fekete, M. A. R. & Cardini, C. E. (1964). *Arch. Biochem. Biophys.* **104**, 173.
Gould, S. E. B. & Rees, D. A. (1965). *J. Sci. Fd. Agric.* **16**, 702.
MacLeod, A. M. (1957). *New Phytol.* **56**, 210.
Murata, T., Sugiyama, T., Minamikawa, T. & Akazawa, T. (1966). *Arch. Biochem. Biophys.* **113**, 34.
Pazur, J. H., Shadaksharaswamy, M. & Meidell, D. (1962). *Arch. Biochem. Biophys.* **99**, 78.
Pridham, J. B. (1958). *Nature, Lond.* **182**, 1687.
Pridham, J. B. & Hassid, W. Z. (1965). *Plant Physiol.* **40**, 984.
Shiroya, T. (1963). *Phytochemistry*, **2**, 33.

A 20

Preprinted from the Journal of the Chemical Society, December 1964, (1051), pages 5486—5488.

1051. *The Condensation of Ferrocenealdehyde with* D-*Glucitol and* D-*Mannitol.*

By A. N. DE BELDER, E. J. BOURNE, and J. B. PRIDHAM.

CYCLIC acetal condensations of ferrocenealdehyde have not been widely investigated. Broadhead and his co-workers [1] found that the aldehyde and ethylene glycol give 2-ferrocenyl-1,3-dioxolan.

We have found that the aldehyde condenses with D-glucitol and D-mannitol to give cyclic acetals. These were prepared by refluxing the reactants in (*a*) *NN*-dimethylformamide–benzene in the presence of traces of toluene-*p*-sulphonic acid, or (*b*) *NN*-dimethylformamide in the presence of phosphoric pentoxide. 2,4-*O*-(Ferrocenylmethylene)-D-glucitol was obtained as a sharply melting product (m. p. 196—198°) provided that water was excluded during the working up of the reaction mixture. Its tetra-acetate was also crystalline. The structure of the glucitol acetal was proved by periodate oxidation. Measurement of periodate uptake was complicated by concomitant degradation of the ferrocenyl group; however, by similar techniques to those employed for the ferrocenyl glucosides,[2] the glycol groups of the glucitol acetal were found to liberate no formic acid but 1 mol. of formaldehyde with the consumption of 1 mol. of periodate. L-Xylose, identified chromatographically and as its di-*O*-benzylidene dimethyl acetal, was found to be the only major product present after periodate oxidation and hydrolysis. Thus ferrocenealdehyde resembles other aldehydes [3] in condensing with D-glucitol at positions 2 and 4 to give the favoured β*C*-ring.

From the reaction with D-mannitol, a mono- and a di-*O*-(ferrocenylmethylene)-D-mannitol were isolated. Their structures were not examined in detail. The monoacetal did not crystallise, although it formed a crystalline tetra-acetate in low yield. The diacetal gave a crystalline diacetate. Periodate oxidation and hydrolysis of the mannitol monoacetal gave erythrose and arabinose (identified by chromatography and electrophoresis), indicating that it was probably a mixture of a 1,3- and 3,4-*O*-substituted mannitol. Goldstein and his co-workers [4] reported that 3,4-di-*O*-methyl-D-mannitol consumes only 1 mol. of periodate, giving an arabinose derivative. These ring systems are also found in the mono-*O*-methylenemannitols isolated by Fletcher and Diehl.[5]

The ferrocenyl acetals were hydrolysed completely in 10 minutes by 0·01N-hydrochloric acid at 25°. Their extreme lability is to be understood if the strong electron-donating properties of the ferrocenyl group [6] assist the unimolecular decomposition of the intermediate conjugate acid.[7,8] The usefulness of such acetals as intermediates in syntheses of derivatives of polyols is reduced, however, by the fact that the acetals are formed in much lower yield from the higher polyols than from ethylene glycol,[1] because of solvent difficulties and probably also of steric factors. The mono-*O*-(ferrocenylmethylene)-hexitols are water-soluble compounds, which may be useful in the pharmaceutical field.

Experimental.—Chromatography solvents. A, Butan-1-ol–ethanol–water (4 : 1 : 5 v/v; organic layer); B, ethyl acetate–acetic acid–water (9 : 2 : 2 v/v); C, phenol saturated with water.
Electrophoresis buffers. E1, 0·1M-Borate, pH 10; E2, 0·05M-Germanate, pH 10.

Ferrocenealdehyde. This was prepared as described by Graham *et al.*[9] and had m. p. 114—118° (lit.,[9] 121°).

2,4-O-*Ferrocenylmethylene*-D-*glucitol.* Ferrocenealdehyde (5·4 g.) in benzene (30 ml.) was added to D-glucitol (4·5 g.) in *NN*-dimethylformamide (30 ml.). After addition of toluene-*p*-sulphonic acid (50 mg.), the mixture was stirred and refluxed (*ca.* 120°) for 10 hr. in a Soxhlet apparatus containing calcium chloride. The solvents were then removed *in vacuo* and after the free acid had been neutralised with sodium methoxide, the residue was extracted with hot ethanol. The extracts were condensed and chromatographed on alumina, ethanol being used as the eluant. The top yellow band was isolated from the extruded column and extracted with ethanol. On concentration, the product crystallised. Three recrystallisations from ethanol gave yellow clusters of the *acetal* (0·5 g.), m. p. 196—198°, $[\alpha]_D^{24}$ −95° (*c* 0·1 in H_2O) (Found: C, 53·9; H, 5·9; ferrocenealdehyde, 58. $C_{17}H_{22}FeO_6$ requires C, 54·0; H, 5·9; ferrocenealdehyde, 57%). Recrystallisation of the acetal from water produced yellow needles, m. p. 168—190°. Intensive drying did not alter the m. p. With acetic anhydride in pyridine the acetal gave 1,3,5,6-*tetra*-O-*acetyl*-2,4-O-(*ferrocenylmethylene*)-D-*glucitol*, m. p. 112—113° (Found: C, 54·8; H, 5·9; Ac, 30·8. $C_{25}H_{30}FeO_{10}$ requires C, 55·0; H, 5·5; Ac, 31·5%).

Determination of the aldehyde content of the acetals. The acetal (2—5 mg.) was dissolved in ethanol in a 50-ml. volumetric flask. N-Sulphuric acid (5 ml.) was added and the solution made up to the mark with ethanol (with cooling). The absorption at 269 mμ was measured rapidly against a control made up by diluting 5 ml. of N-sulphuric acid with ethanol to 50 ml. A calibration curve of aldehyde concentration against absorption was found to be linear within the range 6—60 μg/ml.

Periodate oxidations. The acetals were oxidised in dioxan–0·02M-potassium periodate (2 : 3 v/v) adjusted by preliminary experiments to pH 6. Determination of uptake of oxidant and liberation of formic acid were carried out as described by Fleury and Lange,[10] and Anderson *et al.*,[11] respectively. For the determination of formaldehyde an initial pH 7·5 was used.[12]

Oxidation of the acetal to L-*xylose.* The monoacetal (0·2 g.) was shaken in 0·02M-potassium periodate (50 ml.; initial pH 7) until it dissolved, and the mixture was left 2 hr. The excess of periodate was destroyed with ethylene glycol (0·5 ml.) and after addition of 10N-sulphuric acid (5 ml.) the solution was heated at 90° for 1 hr. The solution was cooled, extracted with chloroform, and neutralised with barium carbonate, the precipitate being washed with hot water. After de-ionisation, the solution was freeze-dried. The presence of xylose was shown by chromatography in solvents A, B, and C. The anhydrous residue was converted into the di-*O*-benzylidene dimethyl acetal (according to the method of Breddy and Jones [13]), m. p. 210·5°, $[\alpha]_D^{22}$ +5° (*c* 0·9 in $CHCl_3$), mixed m. p. 211—212° (lit.,[14] 211—212°).

Reaction between D-*mannitol and ferrocenealdehyde.* D-Mannitol (7·2 g.) and ferrocenealdehyde (19·2 g.) in *NN*-dimethylformamide (20 ml.) were stirred at 70° in the presence of phosphoric pentoxide (0·2 g.) for 15 hr. After being cooled, the mixture was neutralised with sodium methoxide and then separated between chloroform (extract A) and water. The aqueous extract was washed four times with chloroform and then freeze-dried. The freeze-dried residue was chromatographed on a cellulose column, solvent A being used as eluant. The yellow band was eluted and the solvent evaporated. Attempts to crystallise the residue from ethanol or water failed. Finally an aqueous solution was freeze-dried to give the mono-*O*-(ferrocenylmethylene)-D-mannitol fraction as a yellow powder (1·5 g.), $[\alpha]_D^{22}$ +37° (*c* 1·0 in H_2O). The product (0·4 g.) with acetic anhydride–pyridine gave, after two recrystallisations from aqueous ethanol, yellow crystals of a *tetra*-O-*acetylmono*-O-(*ferrocenylmethylene*)-D-*mannitol* (0·1 g.), m. p. 112—113°, $[\alpha]_D^{22}$ +9° (*c* 2·0 in $CHCl_3$) (Found: C, 54·5; H, 5·6. $C_{25}H_{30}FeO_{10}$ requires C, 55·0; H, 5·5%).

Di-O-(*ferrocenylmethylene*)-D-*mannitol.* The chloroform extracts (A) from the monoacetal preparation were condensed and chromatographed on alumina, chloroform being used as the eluant. After the complete elution of the unchanged aldehyde, the column was extruded and the top yellow band was extracted with ethanol. Evaporation of the ethanol and treatment with acetic anhydride–pyridine yielded, after three recrystallisations from chloroform–ethanol, orange-yellow crystals of the *diacetate* of the diacetal (0·3 g.) m. p. 196—200°, $[\alpha]_D^{24}$ −6° (*c* 1·0 in $CHCl_3$) (Found: C, 58·7; H, 5·5; Ac, 15·5; ferrocenealdehyde, 66·3. $C_{32}H_{34}Fe_2O_8$ requires C, 58·4; H, 5·2; Ac, 13·1; ferrocenealdehyde, 65·0%). De-acetylation of this acetate gave, after two recrystallisations from chloroform–hexane, yellow-orange crystals of the *diacetal,*

m. p. 195—200°, $[\alpha]_D^{20}$ +39° (*c* 2·7 in $CHCl_3$) (Found: C, 59·1; H, 5·4; Fe, 18·6. $C_{28}H_{30}Fe_2O_6$ requires C, 58·6; H, 5·3; Fe, 19·4%).

One of us (A. N. de B.) thanks Aspro-Nicholas Ltd. for a grant.

ROYAL HOLLOWAY COLLEGE, UNIVERSITY OF LONDON,
ENGLEFIELD GREEN, SURREY. [*Received, March 26th*, 1964.]

[1] Broadhead, Osgerby, and Pauson, *J.*, 1958, 650.
[2] de Belder, Bourne, and Pridham, *J.*, 1961, 4464.
[3] Barker and Bourne, *J.*, 1952, 905.
[4] Goldstein, Sorger-Domenigg, and Smith, *J. Amer. Chem. Soc.*, 1959, **81**, 444.
[5] Fletcher and Diehl, *J. Amer. Chem. Soc.*, 1952, **74**, 3799.
[6] Arnett and Bushick, *J. Org. Chem.*, 1962, **27**, 111.
[7] Ceder, *Arkiv Kemi*, 1954, **6**, 523.
[8] Leutner, *Monatsh.*, 1935, **66**, 222.
[9] Graham, Lindsey, Parshall, Peterson, and Whitman, *J. Amer. Chem. Soc.*, 1957, **79**, 3416.
[10] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.
[11] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.
[12] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 583.
[13] Breddy and Jones, *J.*, 1945, 738.
[14] Hockett and Schaefer, *J. Amer. Chem. Soc.*, 1947, **69**, 849.

PRINTED IN GREAT BRITAIN BY RICHARD CLAY AND COMPANY, LTD.,
BUNGAY, SUFFOLK.

Preprinted from the Journal of the Chemical Society,
October, 1961, (879), pages 4464—4467.

A21

879. β-*Glucopyranosides of Hydroxymethyl- and Hydroxyethyl-ferrocene.*

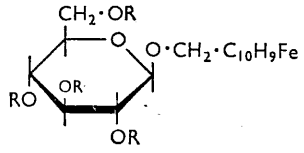
By A. N. de Belder, E. J. Bourne, and J. B. Pridham.

Synthesis and properties of ferrocenylmethyl and ferrocenylethyl β-D-glucopyranoside are described. The sensitivites of the compounds to dilute mineral acid have been investigated.

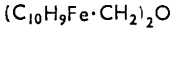
As a first step in the study of carbohydrate derivatives of ferrocene, ferrocenylmethyl β-D-glucopyranoside (Ia) has been prepared by the Koenigs–Knorr [1] reaction under the conditions recommended by Reynolds and Evans.[2]

Condensation of hydroxymethylferrocene with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in the presence of silver oxide and calcium sulphate yielded mainly ferrocenylmethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (Ib), with some di(ferrocenylmethyl) ether (II). Deacetylation gave yellow crystals of the glucoside (Ia) monohydrate. This was also prepared in low yield by reaction of D-glucose with hydroxymethylferrocene in the presence of toluene-*p*-sulphonic acid.[3]

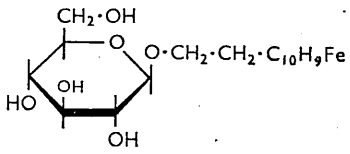
The structure of the glucoside (Ia) was proved by acidic and enzymic hydrolyses, methylation, and periodate oxidation. Potassium metaperiodate, in addition to reacting with the glucosyl residue, degraded the ferrocene nucleus, producing insoluble material which was probably a mixture of ferric hydroxide and ferric periodate or possibly potassium periodate adsorbed on ferric hydroxide. Ferric salts interfered with the periodate consumption (cf. Lang and Faude [4]), so the acetate (Ib) and the glucoside (Ia)



(I) a: R = H
b: R = Ac



(II)



(III)

were oxidised in identical conditions and the periodate uptake by the glucosyl residue of (Ia) and the formaldehyde liberation were calculated by difference. In this way it was shown that the glucosyl group consumed 2 mol. of oxidant and produced no formaldehyde, consistently with a glucopyranoside structure. Methylation of the glucoside, followed by hydrolysis, yielded crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose. Emulsin hydrolysed the glucoside to glucose and hydroxymethylferrocene, and this result together with the specific optical rotation of the compound ($[\alpha]_D^{22}$ −37·7°) confirms the view that the molecule is a β-glucopyranoside.

0·048N-Sulphuric acid rapidly hydrolysed the glucoside at 25°, giving hydroxymethylferrocene, glucose, and di(ferrocenylmethyl) ether (II). The first-order rate constant for this reaction was 8·6 × 10^{-2} min.$^{-1}$ (Table 1), which resembles the value for the hydrolysis

Table 1.

Hydrolysis of the glucoside (Ia; 0·0218M) in 0·048N-H_2SO_4.

Time (min.) ...	0	8	15	21	26	∞
α_D^{25}	−0·28° (calc.)	−0·04°	+0·05°	+0·11°	+0·13°	+0·18° (calc.)
k_1 (min.$^{-1}$)	—	0·088	0·083	0·088	0·084	—

of a furanoside rather than a pyranoside.[5] The nature of the bond fission was therefore investigated by conducting the hydrolysis in the presence of $H_2{}^{18}O$. This indicated that the hydrolysis occurred predominantly by hexosyl–oxygen bond fission (Table 2). Similar results have been obtained by Bunton *et al.*[6,7] with methyl and phenyl glucopyranosides, methyl 2-deoxyglucopyranosides, and maltose. No conclusions can be drawn from the

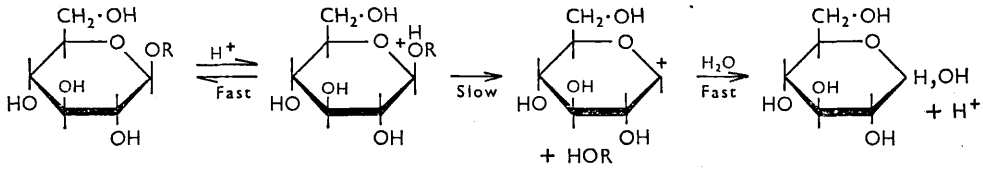
isotope experiments regarding the formation of the ether: it could be formed by direct reaction of a ferrocenylmethyl cation with the glucoside (Ia) or by further reaction of the hydroxymethylferrocene liberated in the hydrolysis.

TABLE 2.

	Fcn·CH_2· OH	Fcn·CH_2·O· CH_2·Fcn	Glucose *	Fcn·CH_2·OH (control)	Glucose (control)	Water
Abundance (atom %) †	0·35	0·25	0·93	0·18 ‡	0·18	1·11
Excess abundance (atom %) ...	0·17	0·07	0·74	0	0	0·93

* Calculated from the observed isotopic ratio on the assumption that only the 1-hydroxyl group was enriched. † ^{18}O atom % abundance is given by $100 - (2R + 1)$ when R is $[C^{16}O_2]/[C^{18}O^{16}O]$. ‡ Normal abundance.

Bunton *et al.*[6,7] have shown that the acidic hydrolysis of a number of glucopyranosides proceeds by the annexed mechanism. The alternative scheme whereby the cyclic oxygen atom is protonated and the carbonium ion is formed by ring-opening between $C_{(1)}$ and this oxygen atom is not favoured.



The rates of hydrolysis of some glucosides[8] increase as the aglycone residue is changed from a primary to a tertiary group. In some respects hydroxymethylferrocene has properties characteristic of a tertiary alcohol. For example, it is readily converted into an ether (II) in the presence of acid[9] and attempts to prepare its toluene-*p*-sulphonate in pyridine produce the 1-ferrocenylmethylpyridinium salt.[10] Bunton *et al.*[7] have recently shown that t-butyl D-glucopyranoside is rapidly hydrolysed by acid, but in this instance by alkyl–oxygen bond fission and not by the hexosyl–oxygen bond fission which occurs predominantly with ferrocenylmethyl β-D-glucopyranoside. Ferrocene has many properties characteristic of a highly activated benzene derivative,[11] so in the case of the glucoside (Ia) the ferrocene nucleus might be expected to increase the basicity of the exocyclic oxygen atom and thereby facilitate formation of the conjugate acid during hydrolysis. Nath and Rydon[12] showed that the introduction of electron-repelling groups into the aromatic ring of phenyl β-D-glucopyranoside increased the lability of this glucoside to acid, but comparatively slightly. Edward[13] suggested that phenyl glucosides are under strain. Thus the resulting lack of planarity may restrict the conjugate displacements in these molecules and the polar influences would then be small. A model of the glucoside (Ia) shows that there is no marked intramolecular steric pressure, and polar influences therefore presumably play the more important role in the stability of this compound to acid.

Ferrocenylethyl β-D-glucopyranoside, also prepared by the Koenigs–Knorr method, was virtually stable to 0·05N-sulphuric acid at 25° and was incompletely hydrolysed by this acid after 20 hr. at 70°. The greater stability of this glucoside (II) than of ferrocenylmethyl β-D-glucopyranoside (Ia) is presumably due to the additional carbon atom in the former which reduces the electrical influence of the ferrocene nucleus.

Further quantitative studies of the substitution of ferrocene and its derivatives may clarify the position and it is obvious that great care must be taken in assigning pyranoside or furanoside structures solely on the basis of stability to acid.

EXPERIMENTAL

Butan-1-ol–ethanol–water (4 : 1 : 5, v/v; organic layer) was used for the paper chromatography of sugars, and a system comprising two organic phases[14] for ferrocene derivatives.

Hydroxymethylferrocene.—This compound was prepared from (ferrocenylmethyl)trimethylammonium iodide,[15] as described by Hauser and Lindsay,[16] and, recrystallised from hexane, had m. p. 75—76° (lit.,[17] 76°).

Hydroxyethylferrocene.—Acetylferrocene was converted into this compound as described by Rinehart, Curby, and Sokol; [18] the product had m. p. 45—47° (lit.,[18] 49—50°) after recrystallisation from ether–hexane.

Ferrocenylmethyl 2,3,4,6-*Tetra*-O-*acetyl*-β-D-*glucopyranoside* (Ib) (*Köenigs–Knorr Method*).—2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4·1 g.) in methylene chloride (30 ml.) was added slowly to a mixture of hydroxymethylferrocene (3·3 g.), silver oxide (9·2 g.), and calcium sulphate (5·0 g.) in methylene chloride (30 ml.), that was then shaken for 18 hr., filtered, and evaporated to dryness. The residue dissolved in hot methanol and on cooling gave a precipitate which when recrystallised from benzene–hexane afforded orange crystals of the *acetate* (Ib) (3·0 g., 54%), m. p. 183—185°, $[\alpha]_D^{21}$ −11·7° (*c* 1·0 in $CHCl_3$) (Found: C, 55·0; H, 5·8; Fe, 10·0; Ac, 31·3. $C_{25}H_{30}FeO_{10}$ requires C, 54·9; H, 5·7; Fe, 10·2; Ac, 31·5%).

Di(ferrocenylmethyl) Ether (II).—Fractionation of the methanolic filtrate from the Koenigs–Knorr reaction [remaining after the precipitation of the acetate (Ib)] on a column of alumina (with benzene, benzene–hexane, and benzene–chloroform) produced crystals of di(ferrocenylmethyl) ether (0·9 g.), m. p. 130—131° (lit.,[19] 129—130°) (Found: C, 63·3; H, 5·5. Calc. for $C_{22}H_{22}OFe_2$: C, 63·8; H, 5·4%).

Ferrocenylmethyl β-D-*Glucopyranoside* (Ia).—The acetate (Ib) (1·0 g.) was treated with 0·2N-sodium methoxide (1 ml.) in methanol–chloroform at room temperature for 24 hr. The resulting water-soluble fraction contained the *glucoside* (Ia) which after one recrystallisation from water was obtained as yellow plates of the monohydrate (0·4 g., 50%), m. p. 135—136°, $[\alpha]_D^{22}$ −37·7° (*c* 1·0 in H_2O) (Found: C, 51·8; H, 6·3; Fe, 14·1%; H_2O, 0·98 mol. $C_{17}H_{22}FeO_6,H_2O$ requires C, 51·5; H, 6·1; Fe, 14·1%).

Ferrocenylmethyl β-D-*Glucopyranoside* (Ia) (*Fischer Method*).—Hydroxymethylferrocene (0·1 g.), D-glucose (0·05 g.), and toluene-*p*-sulphonic acid (5 mg.) in dimethylformamide (5 ml.) were heated for 6 hr. at 80—90°. Fractionation of the mixture by partition chromatography on Whatman No. 3MM paper yielded a small specimen of the glucoside (Ia), m. p. 133—136°.

Hydrolysis of the Glucoside (Ia).—(a) *With Amberlite* IR-120 (H^+ *form*) *resin.* The glucoside (50 mg.) in 90% aqueous ethanol was shaken with the resin for 2 hr. at 80—90°. The resulting glucose and hydroxymethylferrocene were identified by paper chromatography.

(b) *With emulsin.* The glucoside (5 mg.) was incubated with emulsin solution (3 ml.) at 30° for 18 hr., and the products, glucose and hydroxymethylferrocene, were characterised by paper chromatography.

Methylation of the Glucoside (Ia).—Silver oxide (0·5 g.) was added during 30 min., with continual agitation, to the glucoside (0·2 g.) in dimethylformamide (5 ml.) and methyl iodide (1 ml.). Shaking was continued for 18 hr. and the product isolated by partition between water and chloroform. The methylation was repeated and the product isolated as a syrup (0·1 g.) that was dissolved in N-hydrochloric acid (10 ml.); steam was passed through this solution for 30 min. After cooling, hydroxymethylferrocene was removed by extraction with ether. The hydrolysate was then saturated with sodium sulphate and extracted with chloroform. The chloroform solution was decolorised with charcoal, dried ($MgSO_4$), and concentrated to a syrup. 2,3,4,6-Tetra-*O*-methyl-D-glucose (40 mg.), m. p. 96—97°, mixed m. p. 95—97°, was obtained from a light petroleum (b. p. 40—60°) solution of this, and its identity was confirmed by paper chromatography.

Periodate Oxidations.—The glucoside (Ia) (10—20 mg.) was dissolved in dioxan (20 ml.), and 0·02M-potassium metaperiodate (30 ml.; adjusted to pH 7) was added. Samples (3 ml.) were removed at intervals and the periodate uptake and formaldehyde liberation determined by the methods of Fleury and Lange [20] and O'Dea and Gibbons,[21] respectively. The experiment was repeated with the acetate (Ib).

Time (hr.)		5	8	10
Periodate consumed (mol.) :	acetate (Ib)	10·5	11·5	11·5
	glucoside (Ia)	12·0	13·5	13·5

Kinetic Measurements.—The acidic hydrolysis at 25° was followed by dissolving the glucoside (Ia) in water (25 ml.), then adding N-sulphuric acid (1·25 ml.). At intervals samples (5 ml.) were withdrawn, neutralised with 0·05N-ammonia, and centrifuged and the optical rotations of the solutions were then measured. The hydrolysis was carried out with the glucoside at

0·0218M-concentration (Table 1). Errors between calculated and observed values by this procedure were generally less than 4%.

Hydrolysis of Glucoside (Ia) *in the Presence of* $H_2{}^{18}O$.—The glucoside (*ca.* 0·4 g.) was dissolved in ^{18}O-enriched water (30 ml.; 0·8 atom %). N-Sulphuric acid (1·5 ml.) was added. The solution was left at room temperature for 45 min., then extracted with benzene. The aqueous layer was shaken with an excess of barium carbonate, filtered, and treated with Biodeminrolit (carbonate form). After filtration and freeze-drying the resulting glucose crystallised from methanol–water–propan-2-ol (m. p. 149—152°). The benzene layer was washed with water and dried (K_2CO_3). Fractionation on an alumina column yielded hydroxymethylferrocene (40 mg.), m. p. 74—76°, and di(ferrocenylmethyl) ether (50 mg.), m. p. 130—131°.

Control experiments with β-D-glucose and hydroxymethylferrocene were carried out under the same conditions.

All compounds were analysed isotopically as carbon dioxide.[22]

Ferrocenylethyl β-D-*Glucopyranoside* (III).—Hydroxyethylferrocene (0·39 g.) in methylene chloride (3 ml.) was added to 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0·62 g.), silver oxide (0·5 g.), and calcium sulphate (0·5 g.) in methylene chloride (5 ml.), and the mixture was shaken for 20 hr. Filtration followed by removal of the solvent left a residue which was dissolved in methanol. 0·1N-Sodium methoxide (1 ml.) was then added and the products were partitioned between water and chloroform. The aqueous layer was freeze-dried and the residual *glucoside* (0·1 g.) (III) crystallised from water; it had m. p. 174°, $[\alpha]_D^{23} - 28°$ (*c* 0·3 in H_2O) (Found: C, 55·3; H, 6·1. $C_{18}H_{24}FeO_6$ requires C, 55·1; H, 6·2%).

Hydrolysis of Glucoside (III).—(a) *With acid.* The glucoside (10 mg.) was dissolved in 0·05N-sulphuric acid (10 ml.) at 25° and samples (1 ml.) were removed at intervals, neutralised with barium carbonate, and examined for glucose on paper chromatograms. This experiment was repeated at 70°.

(b) *With emulsin.* This was carried out under the conditions described for the hydrolysis of the glucoside (Ia). The resulting glucose and hydroxyethylferrocene were identified by paper chromatography.

We are grateful to Dr. C. A. Bunton for a gift of ^{18}O-enriched water and to Drs. J. Brimacombe, A. B. Foster, and J. Majer for assistance with the isotopic analyses. One of us (A. N. de B.) is indebted to Aspro-Nicholas Ltd. for a grant.

ROYAL HOLLOWAY COLLEGE,
ENGLEFIELD GREEN, SURREY. [*Received, April 19th,* 1961.]

[1] Koenigs and Knorr, *Ber.*, 1901, **34**, 957.
[2] Reynolds and Evans, *J. Amer. Chem. Soc.*, 1938, **60**, 2559.
[3] Combs, McCloskey, Sundberg, and Coleman, *J. Amer. Chem. Soc.*, 1949, **71**, 276.
[4] Lang and Faude, *Z. anorg. Chem.*, 1937, **232**, 271.
[5] Pigman and Goepp. " Chemistry of the Carbohydrates," Academic Press Inc., New York, 1948, p. 206.
[6] Bunton, Lewis, Llewellyn, and Vernon, *J.*, 1955, 4419.
[7] Armour, Bunton, Patai, Selman, and Vernon, *J.*, 1961, 412.
[8] Veibel and Hjorth, *Acta Chem. Scand.*, 1952, **6**, 1353.
[9] Hauser and Cain, *J. Org. Chem.*, 1958, **23**, 2007.
[10] de Belder, Bourne, and Pridham, unpublished results.
[11] Pauson, " Non-Benzenoid Aromatic Compounds," Interscience Publ., Inc., New York, 1959, p. 123.
[12] Nath and Rydon, *Biochem. J.*, 1954, **57**, 1.
[13] Edward, *Chem. and Ind.*, 1955, 1102.
[14] de Belder, Bourne, and Pridham, *Chem. and Ind.*, 1959, 996.
[15] Osgerby and Pauson, *J.*, 1958, 656.
[16] Hauser and Lindsay, *J. Org. Chem.*, 1956, **21**, 382.
[17] Broadhead, Osgerby, and Pauson, *J.*, 1958, 650.
[18] Rinehart, Curby, and Sokol, *J. Amer. Chem. Soc.*, 1957, **79**, 3420.
[19] Graham, Lindsey, Parshall, Peterson, and Whitman, *J. Amer. Chem. Soc.*, 1957, **79**, 3416.
[20] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.
[21] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.
[22] Doering and Dorfman, *J. Amer. Chem. Soc.*, 1953, **75**, 5595.

PRINTED IN GREAT BRITAIN BY RICHARD CLAY AND COMPANY, LTD.,
BUNGAY, SUFFOLK.

REPRINTED FROM CHEMISTRY AND INDUSTRY, 1961, p. 432

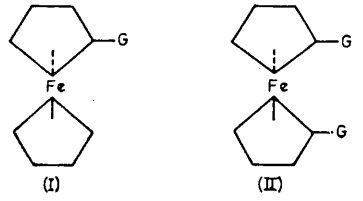
GLUCOSYL DERIVATIVES OF CYCLOPENTADIENE AND CYCLOPENTANE

By A. N. de Belder, E. J. Bourne and J. B. Pridham

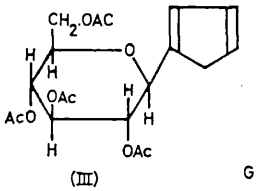
Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey

As a possible intermediate for the preparation of glucosylated ferrocene derivatives (I and II) a glucosylated cyclopentadiene derivative (III) has been prepared.

Freshly distilled cyclopentadiene (11·0 g.) in ether (25 ml.) was added slowly to ethyl bromide (18·0 g.) and magnesium turnings (4·0 g.) in ether (75 ml.).[1] After heating under reflux and stirring for 12 hours, 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (7·0 g.) in ether (30 ml.) was added over 15 minutes and the heating and stirring continued for a further 5 hours. The reaction mixture was then cooled, and poured onto ice-cold dilute acetic acid solution (150 ml.). Dilute alkali and water were used to wash the ether layer, both washings combined with the aqueous layer, and the whole then neutralised and dried in the frozen state. Acetylation of this latter material with acetic anhydride yielded 1-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)cyclopenta-1,3-diene (III) as a syrup (1·5 g.) which was crystallised from isopropanol, m.p. 126–128°, $[\alpha]_D^{24}$ −27° (*c.*, 0·6 in $CHCl_3$), $\lambda_{max.}$ 206 mμ (log ε 3·3) and 247 mμ (log ε 3·8) (Found: C, 57·8; H, 6·55. $C_{19}H_{24}O_9$ requires C, 57·6; H, 6·10%). The cyclopentadiene derivative (III) reacted with maleic anhydride to give an adduct, m.p. 216–217° (Found: C, 56·2; H, 5·24; M, 477. $C_{23}H_{26}O_{12}$ requires C, 55·9; H, 5·30%). The peak at 206 mμ is associated with ester carbonyl absorption and the maximum at 247 mμ results when a cyclic diene possesses a saturated substituent in the 1-position.[2,3] The ultraviolet absorption spectrum together with a molecular weight determination indicated that (III) was a monomer and the infrared spectrum showed a peak at 890 cm.$^{-1}$ which is characteristic of a β-glucopyranosyl configuration.[4]



(I) (II)



(III) G = β-D-GLUCOPYRANOSYL

Hydrogenation of compound (III) with Adams' catalyst gave 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl cyclopentane, m.p. 90–91°, $[\alpha]_D^{20}$ −15° (*c.*, 1·2 in MeOH) (Found: C, 56·99; H, 7·04. $C_{19}H_{28}O_9$ requires C, 56·99; H, 7·05%), and this was oxidised with alkaline permanganate to cyclopentane carboxylic acid which was characterised by infrared spectrophotometry.

All attempts to prepare compounds (I) and (II) by reaction of the anion of the cyclopentadiene derivative (III) with cyclopentadiene in the presence of ferric chloride failed. Steric factors may be involved in this type of reaction as Pauson[5] has reported that highly substituted phenyl derivatives of cyclopentadiene cannot be converted to the corresponding ferrocene derivatives.

The authors are indebted to Aspro-Nicholas Ltd. for provision of a scholarship (to A. N. de B.) and to to Dr. D. Peters for a specimen of cyclopentane carboxylic acid.

Received January 18, 1961

References

1 Grignard & Courtot, *Compt. rend.*, 1914, **158**, 1763
2 Peters, *J. chem. Soc.*, 1959, 1761
3 Powell & Edson, *Anal. Chem.*, 1948, **20**, 510
4 Barker, Bourne & Whiffen, In "Methods of Biochemical Analysis," Vol. III, 1956, p. 229. Editor, D. Glick (New York: Interscience Pub. Inc.)
5 Pauson, *J. Amer. chem. Soc.*, 1954, **76**, 2187

PRINTED BY RICHARD MADLEY LTD., 54 GRAFTON WAY, LONDON, W.1, ENGLAND

REPRINTED FROM CHEMISTRY AND INDUSTRY, 1959, pp. 996—997

PAPER CHROMATOGRAPHY OF FERROCENE AND ITS DERIVATIVES

By A. N. de Belder, E. J. Bourne and J. B. Pridham

Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey

Mixtures of ferrocene derivatives may readily be fractionated by adsorption chromatography, for example on alumina, but partition chromatography, with water as the stationary phase, is generally unsuitable owing to the hydrophobic properties of many of these compounds.

Paper chromatographic methods have now been developed in this laboratory in order to identify ferrocene derivatives in complex reaction mixtures. An organic two-phase solvent system is used, which is essentially the same as that described by Wickberg[1] for the separation of sugar acetates. Strips of Whatman No. 3 paper are dipped twice in a solution of dimethyl sulphoxide in benzene (20% v/v) and the benzene is removed from the papers after each treatment by heating at 80° in an oven for approximately 30 secs. The mixtures for analysis (dissolved in absolute ethanol) are quickly applied to the paper strips which are then developed with light petroleum (b.p. 60–80°) saturated with dimethyl sulphoxide. This solvent has a high rate of movement (20 cm./hour at 20°) and complete resolution of a mixture of ferrocene (I), acetylferrocene (II), ferrocenealdehyde (III), hydroxymethylferrocene (IV) and ferrocenecarboxylic acid (V) can easily be achieved in 2 hours, the R_F values for these compounds being 0·98, 0·62, 0·59, 0·19 and 0·05 respectively.

The majority of ferrocene compounds are highly coloured and are therefore readily located on paper chromatograms. However, for a more positive identification, the chromatograms can be sprayed with a solution of potassium thiocyanate (5% w/v in NHCl) (*cf.* Goldberg[2]). This reagent at room temperature produces an immediate red-brown colouration with compounds (II), (III) and (V) and on heating the chromatograms at 90° for 7–8 minutes compounds (I) and (IV) also give the same colours. Prolonged heating (90° for 15 minutes) causes all the spots to change to yellow-brown. The rate of appearance of the original red-brown colour on the chromatogram can assist in the identification of the derivative. A further useful spray reagent, for the detection of certain ferrocene derivatives, is aqueous sodium periodate solution (1% w/v). Compounds (I), (IV) and (V) are rapidly oxidised by periodate at room temperature to blue-green products; in the case of compound (I), the product is presumably the ferrocinium ion. No colour change is apparent with (II) and (III). If required, the thiocyanate reagent can be applied to chromatograms which have already been sprayed with periodate solution, and this will then give the normal red-brown colour with all the above-mentioned derivatives. With compound (IV) this second colour change is slow and goes via a dark green intermediate.

One of the most difficult compounds to detect on chromatograms which have been developed with the solvent system described is (I) which moves near the solvent front and tends to become dispersed over a large area of paper. It is probably best to rely on the natural colour of this compound for its location and this should be marked as soon as the paper is withdrawn from the chromatography tank.

One of us (A. N. de B.) is indebted to Aspro-Nicholas Ltd., for a research grant.

Received June 15, 1959

References

1 Wickberg, B., *Acta chem. Scand.*, 1958, **12**, 615
2 Goldberg, S. I., *Anal. Chem.*, 1959, **31**, 486

PRINTED BY RICHARD MADLEY LTD., 54 GRAFTON WAY, LONDON, W.1, ENGLAND

PHENOLIC COMPOUNDS

B1 - B21

B1

Reprinted from ANALYTICAL CHEMISTRY, Vol. 29, Page 1167, August 1957

Determination of Phenolic Glycosides and Aglycones on Paper Chromatograms

JOHN B. PRIDHAM[1]

The Institute of Paper Chemistry, Appleton, Wis.

▶ The method was developed in order that small amounts of phenols and phenolic glycosides, present in enzymic digests, could be accurately determined. The technique has been used for the determination of catechol and arbutin over a range of 0 to 100 γ, and saligenin from 0 to 70 γ. The errors in all cases were within ±4%. (p-Hydroxyphenyl)-β-gentiobioside has also been determined in enzymic digests after separation of the components of the digests by paper partition chromatography. Quantitative analysis of mixtures of phenols and phenolic glycosides may be rapidly carried out by the simple procedure described. Only a few microliters of the solutions to be analyzed are required, and application of these solutions to the paper as single spots ensures good chromatographic separations of the components. The completeness of separations may be readily observed in view of the fact that the colorimetric reactions are carried out on the chromatograms.

The methods available for the determination of phenols and their derivatives have recently been reviewed by Clarke and Nord (*5*), Trim (*13*), and Bray and Thorpe (*3*). The analytical principles involved are many and varied, but the majority of techniques have been devised for specific phenolic compounds and little attention has been paid to the quantitative separation and determination of mixtures of phenols. One of the few exceptions, however, is Stone and Blundell's method, where the phenolic aldehydes obtained by alkaline nitrobenzene oxidation of lignin are separated by paper partition chromatography and then determined spectrophotometrically after elution from the paper with ethyl alcohol (*9*). This method has recently been utilized for the determination of phenolic carboxylic acids (*1*).

A good chromatographic spray reagent for phenols is diazotized *p*-nitroaniline (*4*), which has also been used for the determination of *p*-hydroxybenzoic acid and *p*-hydroxybenzamide in the ether extracts of urine (*2*).

In the procedure described herein the author has utilized this reagent, in conjunction with paper partition chromatography, to determine phenolic compounds in mixtures.

The technique used is similar to that described for determination of sugars with *p*-anisidine hydrochloride (*6*). The solution containing the phenols is applied to the chromatographic paper as small discrete spots, and, after development with a suitable solvent, the chromatogram is dried and sprayed with a solution of diazotized *p*-nitroaniline buffered with sodium acetate. The excess moisture is then allowed to evaporate, the spots are cut from the chromatogram, and the color is eluted from the paper with a solution of potassium hydroxide in aqueous methanol. Finally, the absorbance of the resulting solution is measured in a spectrophotometer. The intensity of color bears a linear relationship to the weight of phenolic com-

[1] Present address, Chemistry Department, West Mains Road, The University, Edinburgh 9, Scotland.

Reprinted from ANALYTICAL CHEMISTRY, Vol. 29, Page 1167, August 1957

Determination of Phenolic Glycosides and Aglycones on Paper Chromatograms

JOHN B. PRIDHAM[1]

The Institute of Paper Chemistry, Appleton, Wis.

▶ The method was developed in order that small amounts of phenols and phenolic glycosides, present in enzymic digests, could be accurately determined. The technique has been used for the determination of catechol and arbutin over a range of 0 to 100 γ, and saligenin from 0 to 70 γ. The errors in all cases were within ±4%. (p-Hydroxyphenyl)-β-gentiobioside has also been determined in enzymic digests after separation of the components of the digests by paper partition chromatography. Quantitative analysis of mixtures of phenols and phenolic glycosides may be rapidly carried out by the simple procedure described. Only a few microliters of the solutions to be analyzed are required, and application of these solutions to the paper as single spots ensures good chromatographic separations of the components. The completeness of separations may be readily observed in view of the fact that the colorimetric reactions are carried out on the chromatograms.

THE methods available for the determination of phenols and their derivatives have recently been reviewed by Clarke and Nord (*5*), Trim (*13*), and Bray and Thorpe (*3*). The analytical principles involved are many and varied, but the majority of techniques have been devised for specific phenolic compounds and little attention has been paid to the quantitative separation and determination of mixtures of phenols. One of the few exceptions, however, is Stone and Blundell's method, where the phenolic aldehydes obtained by alkaline nitrobenzene oxidation of lignin are separated by paper partition chromatography and then determined spectrophotometrically after elution from the paper with ethyl alcohol (*9*). This method has recently been utilized for the determination of phenolic carboxylic acids (*1*).

A good chromatographic spray reagent for phenols is diazotized *p*-nitroaniline (*4*), which has also been used for the determination of *p*-hydroxybenzoic acid and *p*-hydroxybenzamide in the ether extracts of urine (*2*).

In the procedure described herein the author has utilized this reagent, in conjunction with paper partition chromatography, to determine phenolic compounds in mixtures.

The technique used is similar to that described for determination of sugars with *p*-anisidine hydrochloride (*6*). The solution containing the phenols is applied to the chromatographic paper as small discrete spots, and, after development with a suitable solvent, the chromatogram is dried and sprayed with a solution of diazotized *p*-nitroaniline buffered with sodium acetate. The excess moisture is then allowed to evaporate, the spots are cut from the chromatogram, and the color is eluted from the paper with a solution of potassium hydroxide in aqueous methanol. Finally, the absorbance of the resulting solution is measured in a spectrophotometer. The intensity of color bears a linear relationship to the weight of phenolic com-

[1] Present address, Chemistry Department, West Mains Road, The University, Edinburgh 9, Scotland.

pound over a range of 0 to 100 γ for catechol and arbutin, and from 0 to 70 γ for saligenin.

The method has found application for the determination of (*p*-hydroxyphenyl)-β-gentiobioside (*7*) in enzymic digests, which in addition contain arbutin and quinol.

The method might also be used for the determination of many other phenols and derivatives, if they are capable of coupling with diazotized *p*-nitroaniline, and if no decomposition occurs during the extraction with methanolic potassium hydroxide solution.

REAGENTS

Diazotized *p*-Nitroaniline Solution. This spray reagent is made up as described by Swain (*10*).

To a solution of *p*-nitroaniline in 2*N* hydrochloric acid (0.5% w./v.; 5 ml.) is added aqueous sodium nitrite solution (5.0% w./v.; 0.5 ml.) followed by sodium acetate buffer (20% w./v.; 15 ml.).

These reagents are stable, but the diazotized *p*-nitroaniline is unstable and should be freshly prepared just prior to use, although it may be stored in a refrigerator for 1 to 2 hours after preparation.

Eluting Reagent. The solution used contains potassium hydroxide (0.2% w./v.) in aqueous methanol (95% v./v.).

For the determination of catechol the composition of eluent is modified by decreasing the amount of methanol in the aqueous solution from 95 to 80%.

PROCEDURE

Suitable volumes of the phenol solutions are spotted onto paper chromatograms (Whatman No. 1 paper) with an ultramicroburet. Three or four spots of standard phenol solutions, the components of which correspond qualitatively to those in the unknown, are also applied to the chromatograms. The standard solutions are spotted in amounts ranging from 0 to 100 γ for arbutin and catechol and from 0 to 70 γ for saligenin.

Development of the chromatograms is effected with a suitable solvent, such as 1-butanol–pyridine–water (6:4:3 v./v.), and the papers are then allowed to dry in the air. The chromatograms are next sprayed, as uniformly as possible, with the diazotized *p*-nitroaniline solution and the excess moisture is allowed to evaporate from the paper. The resulting orange-yellow spots, together with suitable blanks, are cut out with scissors, the areas of the pieces of paper being kept constant and as small as possible.

Elution of the colored compounds from the paper is carried out by mechanical shaking in test tubes with the aqueous methanolic potassium hydroxide solution, the volume used varying with the phenol being determined. For arbutin and catechol 3 ml. are used, and for saligenin, 5 ml. The blanks are eluted in a similar manner.

The absorbances of the colored solutions are measured in a Beckman spectrophotometer (Model DU, 1-cm. cell), the red color given by saligenin being diluted ten times before measurement.

Absorbance measurements are made at the following wave lengths: arbutin, 458 mμ; catechol, 470 mμ; and saligenin, 495 mμ.

The values obtained for the standard determinations are plotted against the weights of phenols and the resulting graphs are used to determine the unknowns.

The rate of formation of (*p*-hydroxyphenyl)-β-gentiobioside was studied by withdrawing 1-ml. samples from the enzymic digests at varying time intervals. Each sample, after withdrawal, was immediately deproteinized by shaking with Sevag's reagent (chloroform–*n*-amyl alcohol; 5:2 v./v.) (*8*) and suitable volumes of the top layer were then spotted onto paper chromatograms. These were developed with the 1-butanol–pyridine–water solvent and the (*p*-hydroxyphenyl)-β-gentiobioside was then determined by the procedure described, using arbutin as the standard for comparison.

The weights of (*p*-hydroxyphenyl)-β-gentiobioside present were calculated on the assumption that the stoichiometry of the reactions of diazotized *p*-nitroaniline with arbutin and (*p*-hydroxyphenyl)-β-gentiobioside, respectively, were similar, and that equivalents of the azo dyes thus formed gave the same color intensity.

RESULTS AND DISCUSSION

Azo dyes behave as indicators, and, in general, those formed by coupling diazotized *p*-nitroaniline with phenols

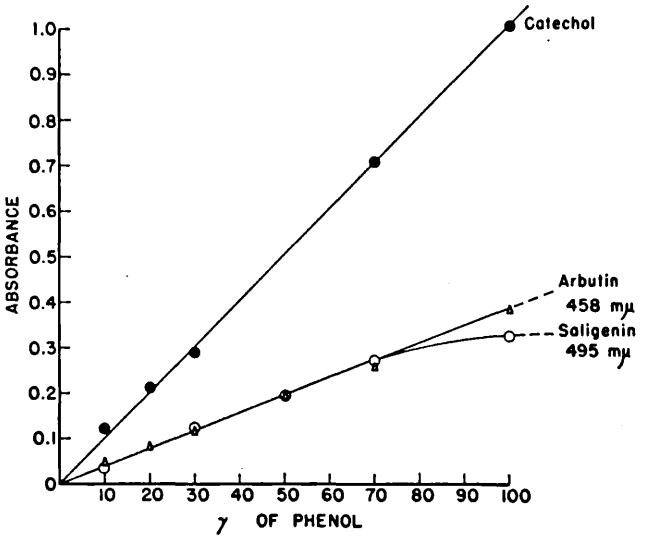


Figure 1. Increase in absorbance of azo dyes with increase in weight of catechol, arbutin, and saligenin

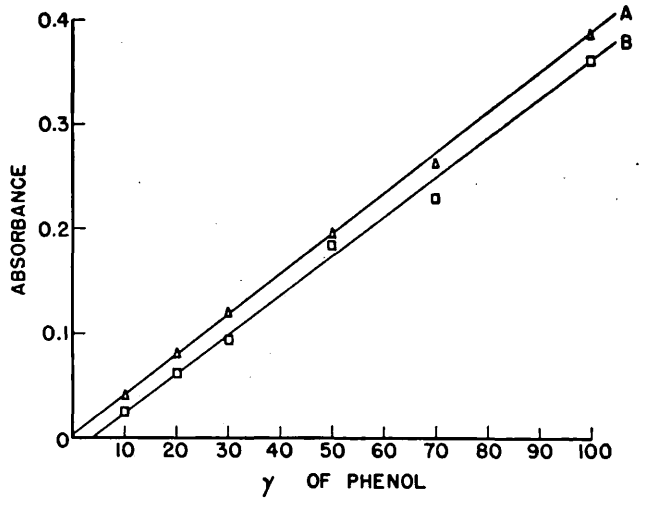


Figure 2. Stability of azo dye formed by reaction of diazotized *p*-nitroaniline with arbutin

A. At zero time
B. After 2 hours and 72 hours

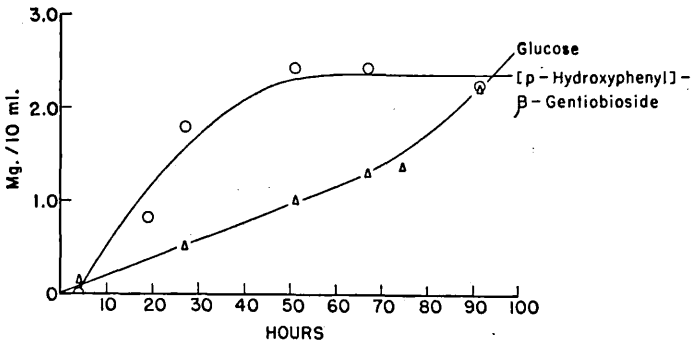


Figure 3. Formation of (p-hydroxyphenyl)-β-gentiobioside and liberation of glucose

Arbutin incubated with β-glucosidase preparation from *Populus grandidentata*

are orange-colored under acidic conditions. If the pH is increased, however, a color change results, which often aids in the identification of certain phenols. These dyes in alkaline solution usually exhibit maximum absorption peaks at a higher wave length than when they are in acid solution and therefore, if necessary, the absorbance of the former can be readily measured in a simple colorimeter. For these reasons the quantitative relationship between the absorbance of the azo dyes and the weights of phenols was examined in alkaline rather than in acid solution. Attempts to bring about the color change by spraying the chromatograms with alkali were abandoned, as this invariably resulted in the colors streaking down the paper. The alkali was therefore incorporated into the eluting reagent.

In the case of catechol, some difficulty was experienced with the elution of the azo dye from the paper. By increasing the water content of the eluting reagent, however, this was overcome.

Standard curves for arbutin, saligenin, and catechol are shown in Figure 1. The relationship between weight of phenol and absorbance was linear from 0 to 100 γ for catechol and arbutin and 0 to 70 γ for saligenin, and the errors were within ±4%.

The stability of the azo dyes formed with the above phenols appeared to be high. The absorbance of the blanks, however, gradually increased over a period of 2 to 4 hours and then remained steady. This gave an apparent decrease, with time, in the intensity of the dye solutions, the standard curves remaining parallel but below the original curves, and no longer passing through the origin (Figure 2). This, however, is of little importance as far as the accuracy of the method is concerned, as the determination of standard phenols together with the unknowns on the same paper chromatogram alleviates any error which might arise from this phenomenon.

The general technique has also been applied to the determination of sugars with *p*-anisidine hydrochloride (*6*) and amino acids with ninhydrin (*11*, *12*). It is rapid, and a useful procedure for the quantitative study of mixtures of compounds of biochemical interest. This is well illustrated in Figure 3, based on a study of the rate of formation of (*p*-hydroxyphenyl)-β-gentiobioside and the simultaneous liberation of glucose when arbutin is incubated with a β-glucosidase preparation from the cambial region of *Populus grandidentata*.

PRINTED IN U. S. A.

LITERATURE CITED

(1) Beyer, D. L., Pearl, I. A., Institute of Paper Chemistry, Appleton, Wis., unpublished results, 1956.
(2) Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K., Wood, P. B., *Biochem. J.* **52**, 416 (1952).
(3) Bray, H. G., Thorpe, W. V., in Glick's "Methods of Biochemical Analysis," Vol. **1**, p. 27, Interscience, New York, 1954.
(4) Bray, H. G., Thorpe, W. V., White, K., *Biochem. J.* **46**, 271 (1950).
(5) Clarke, D. D., Nord, F. F., in Paech and Tracey's "Modern Methods of Plant Analysis," Vol. **3**, p. 332, Springer-Verlag, Berlin, 1955.
(6) Pridham, J. B., ANAL. CHEM. **28**, 1967 (1956).
(7) Pridham, J. B., unpublished results, 1955.
(8) Sevag, M. G., Lackman, D. B., Smolens, J., *J. Biol. Chem.* **124**, 425. (1938).
(9) Stone, J. E., Blundell, M. J., ANAL. CHEM. **23**, 771 (1951).
(10) Swain, T., *Biochem. J.* **53**, 200 (1953).
(11) Thompson, J. F., Stewart, F. C., *Plant Physiol.* **26**, 421 (1951).
(12) Thompson, J. F., Zacharius, R. M., Stewart, F. C., *Ibid.*, **26**, 375 (1951).
(13) Trim, A. R., in Paech and Tracey's "Modern Methods of Plant Analysis," Vol. **2**, p. 295, Springer-Verlag, Berlin, 1955.

RECEIVED for review December 1, 1956. Accepted March 21, 1957.

B2

(*Reprinted from Nature, Vol.* 182, *pp.* 795–796, *Sept.* 20, 1958)

Metabolism of Phenolic Compounds by the Broad Bean *Vicia faba*

PHENOLIC glycosides are common constituents of plant tissues but little is known about the biosynthesis of the glycosidic bond in these compounds. Bourquelot and Hérissey synthesized (*o*-hydroxybenzyl)-β-glucoside by incubating glucose with saligenin in the presence of emulsin[1], but it is unlikely that transglycosylation of this type is an important mechanism for the biosynthesis of glycosides *in vivo*. The formation of aryl glucosides by leaf disks infused with various phenols has been reported by Miwa *et al.*[2] and it appears that glucose-1-phosphate is involved in these reactions.

The stems of young bean shoots were immersed in aqueous solutions containing glucose (0·5 per cent (w/v) and a phenol (0·5 per cent w/v) and after 48 hr. the tissues were extracted with methanol and the extracts examined on paper chromatograms (butan-1-ol/ethanol/water (4 : 1 : 5, organic layer) solvent: ammoniacal silver nitrate[3], diazotized *p*-nitroaniline/sodium hydroxide[4] and periodate–permanganate[5] spray reagents). Some of the products were isolated in small amounts by chromatography on Whatman No. 3 paper and afterwards hydrolysed by an emulsin preparation.

The feeding of quinol and phloroglucinol gave rise to products which co-chromatographed with arbutin (R_F 0·44) and phlorin (R_F 0·35) respectively, and gave the same colour reactions with the diazo reagent as the authentic glycosides. Similarly, the compound obtained by feeding saligenin was indistinguishable from (*o*-hydroxybenzyl)-β-glucoside and hydrolysis with emulsin yielded saligenin and glucose, this being further evidence for the presence of a β-glycosidic linkage. No evidence for the formation of salicin ((*o*-hydroxymethylphenyl)-β-glucoside) from saligenin could be obtained.

Resorcinol, catechol and hydroxyhydroquinone all gave single products (R_F values 0·51, 0·64 and 0·43 (0·33 on phosphate-buffered paper) respectively), which reacted with the diazo- and periodate-permanganate reagents. Hydrolysis with emulsin in each case produced glucose together with the phenol which had been administered to the shoot.

These results suggest that the mono-β-glucosides of catechol and resorcinol had been produced. The

product obtained from hydroxyhydroquinone was probably (3,4-dihydroxyphenyl)-β-glucoside, as it showed a decreased rate of movement when subjected to chromatography on buffered paper, this being evidence for the presence of an *ene-diol* grouping[6]. Small amounts of an electrophoretically and chromatographically identical compound were also formed when young bean shoots were allowed to take up a solution of arbutin (1 per cent w/v). In this case, the arbutin was presumably hydroxylated by polyphenol oxidase.

Glucoside formation could not be demonstrated when phenol was used as a substrate and only traces of possible glucosides were produced using pyrogallol.

The biosynthesis of the glucosides was confined chiefly to the leaves and the activity was completely destroyed by grinding these tissues in the presence of 0·1 *M* phosphate buffer (*p*H 6·0). The synthetic activity of the isolated stems was negligible.

During the preparation of this communication, Hutchinson, Roy and Towers[7] reported glucoside formation by apple-leaf and leaves from a number of other plant species, when fed with phenols in the presence of D-glucose-^{14}C.

This study is being continued to determine the exact mechanism of formation of glucoside in the plant and to examine the biosynthesis and metabolism of the more complex phenolic glycosides containing two or more sugar units.

The interest shown by Prof. E. J. Bourne is very much appreciated.

J. B. PRIDHAM

Department of Chemistry,
Royal Holloway College
(University of London),
Englefield Green, Surrey. July 9.

[1] Bourquelot, E., and Hérissey, H., *C.R. Acad. Sci., Paris*, **156**, 1790 (1913).
[2] Miwa, T., Nakamura, S., and Shibata, A., *Kôso Kagaku Shinpojiuma*, **12**, 48 (1957); *Chem. Abstr.*, **52**, 1314 (1958).
[3] Partridge, S. M., *Biochem. J.*, **42**, 238 (1948).
[4] Bray, H. G., Thorpe, W. V., and White, K., *Biochem. J.*, **46**, 271 (1950).
[5] Lemieux, R. V., and Bauer, H. F., *Anal. Chem.*, **26**, 920 (1950).
[6] Swain, T., *Biochem. J.*, **53**, 200 (1953).
[7] Hutchinson, A., Roy, C., and Towers, G. H. N., *Nature*, **181**, 841 (1958).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

PAPER ELECTROPHORESIS AND PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS

J. B. PRIDHAM

Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey (Great Britain)

The introduction of new analytical techniques, and in particular chromatography, has been largely responsible for the rapid increase, during the last decade, of our knowledge of the biochemistry of phenolic compounds.

The literature on the chromatography of phenolic compounds is extensive but good reviews of developments, up to 1956, are available (BRAY AND THORPE[1]; CLARKE AND NORD[2]; LEDERER AND LEDERER[3]). Amongst the more recent publications in this field is a comprehensive study by REIO[4] of the identification and separation of phenols by paper chromatography.

By comparison with chromatographic methods, the paper electrophoresis of phenolic compounds has been neglected. MICHL[5] has investigated the electrophoresis of borate complexes of *o*-dihydric phenols and COULSON AND EVANS[6] have used borate buffers for their studies with phenolic carboxylic acids and other phenolic derivatives. SHIMOMURA[7] and BERBALK AND SCHIER[8] have also reported electrophoretic studies with phenols.

The present enquiry was undertaken in connection with work on the biosynthesis of phenolic glycosides by plants (PRIDHAM[9]) in an endeavour to supplement existing techniques for the identification and separation of small amounts of phenolic materials. Particular attention has been paid to the use of molybdates as reagents both for the detection and the separation of *o*-dihydroxy compounds by paper electrophoresis and paper chromatography, and the electrophoretic behaviour of phenolic compounds in several different buffer systems has been examined.

EXPERIMENTAL

Molybdate complex formation

The ability of various phenolic compounds to form coloured molybdate complexes was tested by spotting their solutions on to strips of Whatman No. 3 paper which had previously been dipped in a 0.1 *M* sodium molybdate solution, blotted with filter paper, then air-dried. The rapid development of an intense orange-brown colour was taken as a positive indication of complex formation.

Paper electrophoresis

The paper electrophoresis apparatus used for these experiments was built according to a design kindly provided by Dr. D. GROSS of Tate & Lyle, Ltd., and the individual

References p. 611.

runs on Whatman No. 3 paper were continued for 1 ½–2 hours at 22 V/cm. Diazotised *p*-nitroaniline solution (as prepared by SWAIN[10]) followed by *N* NaOH solution, was used to locate the phenols on the paper, and all mobilities were calculated as M_{SA} values, that is, relative to the mobilities of salicylic acid (*o*-hydroxybenzoic acid), 2,3,4,6-tetra-O-methyl-D-glucose being used as a zero marker. *p*-Anisidine hydrochloride (HOUGH, JONES AND WADMAN[11]) was used to locate this carbohydrate derivative. When using alkaline buffers it was necessary to expose the zero marker strips to moist hydrogen chloride gas prior to spraying with the *p*-anisidine hydrochloride reagent. The "absolute mobility" of *o*-hydroxybenzoic acid in each buffer was also calculated.

Buffers

0.2 *M* acetate (pH 5.2)
$8.1 \cdot 10^{-3}$ *M* molybdate (pH 5.2)*
0.2 *M* phosphate (pH 7.2)
0.2 *M* borate (pH 10.0)
0.1 *M* glycine (pH 10.0)

Paper partition chromatography

The descending method of chromatography was adopted and the solvent systems used were butan-1-ol–ethanol–water (4:1:5 v/v; organic layer) and ethyl acetate–acetic acid–water (9:2:2 v/v). Plain and molybdate-treated Whatman No. 3 papers were used for these experiments and diazotised *p*-nitroaniline/NaOH as the spray reagent. The rates of movement of the phenols were recorded as R_F values.

RESULTS AND DISCUSSION

Spot tests with approximately one hundred phenolic compounds using the molybdate-treated paper showed that the presence of an *o*-dihydroxy or *ene-diol* grouping was essential for the formation of a coloured molybdate complex. The test substances, in addition to simple phenols, included phenolic carboxylic acids and nitro- and amino-phenols. Particular attention was paid to phenols having hydroxyl groups *ortho* to other substituents. Tests were also negative with a large number of carbohydrate derivatives, but L-ascorbic acid, an *ene-diol* compound, gave a strong positive reaction.

RILEY[12] has used phosphomolybdic acid followed by ammonia as an unspecific chromatographic spray reagent to detect polyhydric phenols and recently COULSON AND EVANS[6] have used a molybdate reagent to detect *o*-dihydric phenols. With this latter method, however, the chromatograms have to be sprayed with three different solutions. On the basis of the present work it would appear that sodium molybdate alone is a highly specific reagent for *o*-dihydroxy compounds and in this laboratory an aqueous solution (0.1 *M*) has been used successfully as a chromatographic spray reagent. The immediate appearance of orange-brown spots after spraying with this

* $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1% w/v) in 0.002 *N* H_2SO_4.

References p. 611.

reagent is a clear indication of *o*-dihydric phenols. Other phenolic compounds may react slowly to give coloured spots but this only occurs several hours after spraying.

Paper electrophoresis

The results of the paper electrophoresis study are given in Table I, which shows the relative mobilities of a number of phenolic compounds using various buffer systems. In some instances the compounds "tailed" badly (perhaps due to oxidation) and this has been indicated in the table. Using an acetate buffer (pH 5.2) it was observed that

TABLE I

RELATIVE ELECTROPHORETIC MOBILITIES OF PHENOLIC COMPOUNDS EXPRESSED AS M_{SA} VALUES

$$M_{SA} = \frac{\text{distance moved by phenolic compound}}{\text{distance moved by } o\text{-hydroxybenzoic acid}}$$

Compound	M_{SA} values				
	Acetate pH 5.2	*Molybdate pH 5.2*	*Phosphate pH 7.2*	*Borate pH 10.0*	*Glycine pH 10.0*
Quinol	0	0	0.01	**	**
Catechol*	0	1.07	0.01	0.67	0.51
Resorcinol	0	0	0.01	0.35	0.44
Phloroglucinol	0	0	0.01	1.27	1.05
Pyrogallol*	0	0.98**	0.01	0.75**	**
Hydroxyquinol*	0	1.07	1.25**	1.20**	1.16**
3,4-Dihydroxyphenylalanine*	0	0.81	0.01	0.72	**
o-Hydroxybenzoic acid	1.00	1.00	1.00	1.00	1.00
m-Hydroxybenzoic acid	0.81	0.79	0.85	1.05	0.95
p-Hydroxybenzoic acid	0.64	0.70	0.77	1.21	1.10
Protocatechuic acid*	0.50	1.00	0.67	1.19	1.02
Gallic acid*	0.45	1.03	0.59	1.20	0.93
p-Coumaric acid	0.34	0.44	0.58	0.98	0.86
Caffeic acid*	0.20	0.79	0.50	1.04	1.05
Ferulic acid	0.21	0.21	0.42	0.87	0.77
Chlorogenic acid*	0.33	1.03	0.44	0.83	0.74
p-Hydroxybenzaldehyde	0	0	0.27**	0.84**	0.79**
Vanillin	0	0	0.27	0.79	0.76
Syringaldehyde	0	0	0.21	0.71	0.72
Umbelliferone	0	0	0.04	0.76	0.63
4-Methyl-umbelliferone	0	0	0	0.52	0.50
4-Methyl-aesculetin*	0	0.61	0.11	0.48	0.27
Arbutin	0	0	0	0.22	0.09
Aesculin	0	0	0.26	0.51	0.24
Phloridzin	0	0	0.08	0.42	0.14
Catechin*	0	0.23	0	0.65	0.63
Quercetin*	0	0.05	0	0.21	0
Myricetin*	0	0	0	0.18	0
Luteolin*	0	0	0	0.11	0
Kaempherol	0	0	0	0.12	0
Genistein	0	0	0.01	0.25	0.16
Irigenin	0	0	0.04	0.52	0.35
"Absolute mobilities" of *o*-hydroxybenzoic acid (cm/h/1200 V)	8.8	8.8	8.0	9.7	10.8

* *o*-Dihydroxy compounds.
** Compounds decomposed or "tailed".

References p. 611.

phenolic groups were insufficiently ionised to allow migration but compounds possessing carboxyl groups moved rapidly towards the anode. The flavonoids, which were examined, were virtually insoluble in this buffer. With regard to the phenolic carboxylic acids, there appeared to be no simple correlation between their mass/charge ratios and relative mobilities. The relative mobilities observed using the molybdate buffer (pH 5.2) were, in many cases, similar to those obtained with the acetate buffers. Compounds possessing *o*-dihydroxy groupings, however, formed coloured complexes immediately the molybdate buffer was applied to the paper and these, with the exception of the flavonoid derivatives, migrated rapidly towards the anode. The flavonoids and flavonoid–molybdate complexes (with the exception of catechin and possibly quercetin) again appeared to be insoluble in this acidic solution.

The effect of complex formation on mobility is well illustrated by a comparison of the M_{SA} values of catechol and resorcinol in the two acidic buffers.

At pH 7.2 (phosphate buffer) the mobilities of the phenolic carboxylic acids were again, in general, much greater than those of compounds possessing only phenolic groups, but at this pH some movement of many of the latter compounds was observed. Their relative mobilities, however, were normally small and under these conditions it must be assumed that the phenolic hydroxyl groups were only weakly dissociated.

In more strongly alkaline solutions some movement was noted with the majority of phenolic compounds which were examined. In general, with both glycine and borate (pH 10.0) buffers an increase in molecular weight with constant charge retarded the rate of movement. The latter was clearly shown by the phenolic aldehydes where the relative mobilities were in the following order: *p*-hydroxybenzaldehyde > vanillin > syringaldehyde. The high mobilities of the monohydric phenolic aldehydes and also the coumarin derivatives, in alkaline buffers, were rather surprising and suggested that, possibly, these compounds in some way reacted with the basic components of the buffers.

In borate buffer, evidence of complex formation by the *o*-dihydroxy compounds was again obtained, although the results were not so marked as with the molybdate buffer. The borate complexes with *o*-dihydric phenols are presumably similar to those formed with glycols. The behaviour of the flavonoid and trihydroxybenzene derivatives was unpredictable.

At these higher pH values the phenolic hydroxyl groups were highly dissociated and had a marked influence on mobilities. This is apparent when the rates of movement of the phenolic acids in alkaline and acidic buffers are compared. In the majority of cases the mobilities of these compounds were greater in alkaline solutions than in acid because of the increased charge due to hydroxyl dissociation. The behaviour of the isomeric monohydroxybenzoic acids in borate buffer is particularly interesting. Their relative mobilities were in the following order: *para* > *meta* > *ortho*, this being a reversal of the mobilities found with buffers of pH 5.2 and 7.2. At these lower pH values the mobilities of the monohydroxybenzoic acids bore a positive relationship to the dissociation constants, but it is difficult to explain the movement of these compounds in borate buffer.

References p. 611.

In glycine buffer the order of the mobilities of the monohydroxybenzoic acids differed from those in borate and all other buffers which were examined, the M_{SA} values for the *ortho*, *meta* and *para* derivatives being 1.00, 0.95 and 1.10 respectively. The differences between the mobilities of these three acids in the glycine buffer were small but nevertheless definite.

In general it would appear that the rate of movement of phenolic compounds on electrophoretograms is dependent on three main factors; the molecular weight, the degree of dissociation of functional groups, and the ability to form charged complexes with the components of the electrolyte solutions. The latter, in the case of borate and molybdate buffers is governed by the stereochemistry of the phenol. Other factors, such as hydration and distribution of charge, probably also influence mobilities.

This investigation has shown that paper electrophoresis is a valuable tool for the examination of mixtures of phenolic compounds such as those commonly found in extracts of plant tissues. The components of these mixtures which are often present only in trace quantities can normally be identified accurately by comparing their electrophoretic behaviours with those of known specimens, in several different buffers. It is advisable to make a direct comparison of authentic and unidentified specimens on the same electrophoretogram, rather than a comparison of mobilities with those values recorded in the literature. When authentic specimens for comparison are not available, or a new phenolic compound has been isolated, paper electrophoresis can assist in structural determinations. For example, carboxyl and *o*-dihydroxy groupings can be detected quite readily. Paper electrophoresis of relatively high molecular weight compounds, such as phenolic glycosides and flavonoids, is of limited value as their mobilities are usually small, unless strongly ionised groups are present. Some separation of these compounds can be achieved by the use of buffers with high pH values provided the compounds are not alkali-labile.

Paper partition chromatography

WACHTMEISTER[13] and SWAIN[10] have used borate buffered paper for the chromatography of phenolic compounds and, in particular, to detect *o*-dihydric phenols. The movement of these latter compounds was retarded by borate owing to complex formation and similar results have now been obtained using molybdate-treated paper (Table II). The advantage of using molybdate-treated paper is that *o*-dihydroxy compounds form coloured complexes which generally move as discrete spots and spray reagents are therefore not required. Borate complexes are colourless. It was observed that the retardation of movement of *o*-dihydroxy compounds by molybdate was, in general, more marked in the neutral solvent than in the acidic solvent. Using sodium borate-treated papers alone it may be difficult to distinguish between *o*-dihydroxy compounds and phenolic carboxylic acids in neutral solution, as the latter compounds could be retarded owing to the formation of sodium salts. However, by using molybdate this difficulty is overcome as only the *o*-dihydric phenols give the coloured complexes.

Paper chromatography using molybdate-treated paper would appear to offer a

References p. 611.

TABLE II

PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS USING PLAIN AND MOLYBDATE-TREATED PAPERS WITH ACIDIC AND NEUTRAL SOLVENTS

Compound	R_F values			
	Butan-1-ol–ethanol–water solvent		*Ethyl acetate–acetic acid–water solvent*	
	Plain paper	*Molybdate-treated paper*	*Plain paper*	*Molybdate-treated paper*
Phenol	0.94	0.97	0.96	0.91
Quinol	0.87	0.91	0.87	0.68**
Catechol*	0.88	0.38	0.88	0.60
Resorcinol	0.88	0.90	0.88	0.78
Phloroglucinol	0.76	0.76	0.74	0.84
Pyrogallol*	0.77	0.02	0.79	0.25
Hydroxyquinol*	0.74	0.03	0.74	0.34
3,4-Dihydroxyphenylalanine*	0.08	0	0.19**	0.01
o-Hydroxybenzoic acid	0.13	0.15	0.17	0.29
m-Hydroxybenzoic acid	0.25	0.33	0.95	0.92
p-Hydroxybenzoic acid	0.22	0.26	0.94	0.92
Protocatechuic acid*	0.82	0.06	0.86	0.24
Gallic acid*	0.58**	0	0.65	0.11
p-Coumaric acid	0.36	0.36	0.92	0.81
Caffeic acid*	0.68	0.01	0.83	0.44
Ferulic acid	0.23	0.25	0.92	0.90
Chlorogenic acid*	0.19**	0	0.59	0.01
4-Methyl-aesculetin*	0.76	0.21	0.83	0.26
Catechin*	0.63**	0.15	0.68	0.33
Quercetin*	0.14**	0.03	0.84	0.49**
Genistein	0.71**	0.78**	0.98	0.98
Irigenin	0.59**	0.65**	0.97	0.98

* *o*-Dihydroxy compounds.
** Compounds gave elongated spots.

rapid method to distinguish between *o*-dihydroxy compounds, and other phenols, and in conjunction with the electrophoretic techniques, which have been described, a great deal can be done towards the elucidation of the complete structures of unidentified phenolic compounds.

ACKNOWLEDGEMENTS

The electrophoresis apparatus was built with the aid of a grant from the Royal Society. The author wishes to thank Professor E. J. BOURNE for his advice and encouragement, and Dr. J. W. T. SEAKINS and Dr. T. SWAIN for kindly providing some of the specimens.

SUMMARY

An aqueous solution of sodium molybdate has been used as a specific reagent for the detection of phenolic compounds with *o*-dihydroxy groupings.

The paper electrophoretic behaviour of phenolic compounds has been studied using a series of different buffers with pH values ranging from 5.2 to 10.0. *o*-Dihydroxy compounds can readily be detected by the use of ammonium molybdate buffer, and

References p. 611.

other structural features can be revealed by the choice of suitable electrolyte solutions.

Paper partition chromatography of phenolic compounds using molybdate-treated paper is described.

REFERENCES

1 H. G. BRAY AND W.V.THORPE, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 1, Interscience Publishers Inc., New York, 1954, p. 27.
2 D. D. CLARKE AND F. F. NORD, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. 3, Springer-Verlag, Berlin, 1955, p. 332.
3 E. LEDERER AND M. LEDERER, *Chromatography*, Elsevier, Amsterdam, 1957, p. 162.
4 L. REIO, *J. Chromatog.*, 1 (1958) 338.
5 H. MICHL, *Monatsh.*, 83 (1952) 737.
6 C. B. COULSON AND W. C. EVANS, *J. Chromatog.*, 1 (1958) 374.
7 O. SHIMOMURA, *J. Chem. Soc., Japan*, 76 (1955) 277.
8 H. BERBALK AND O. SCHIER, *Monatsh.*, 85 (1955) 146.
9 J. B. PRIDHAM, *Nature*, 182 (1958) 795.
10 T. SWAIN, *Biochem. J.*, 53 (1953) 200.
11 L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702.
12 R. F. RILEY, *J. Am. Chem. Soc.*, 72 (1950) 5782.
13 C. A. WACHTMEISTER, *Acta Chem. Scand.*, 5 (1951) 976.

Received February 2nd, 1959

Printed in The Netherlands

B4

[*Reprinted from the Proceedings of the Biochemical Society*, 23 *January* 1960.
Biochem. J., 1960, Vol. 74, No. 3, 42P.]

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

By J. B. PRIDHAM and M. J. SALTMARSH. (*Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey*)

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.

We thank Professor E. J. Bourne for his advice and encouragement and one of us (M.J.S.) is indebted to the D.S.I.R. for a scholarship.

REFERENCES

Baruah, P. & Swain, T. (1957). *Biochem. J.* **66**, 321.
Cardini, C. E. & Leloir, L. F. (1957). *Cienc. e Invest.* **13**, 514.
Hutchinson, A., Roy, C. & Towers, G. H. N. (1958). *Nature, Lond.*, **181**, 841.
Pridham, J. B. (1958). *Nature, Lond.*, **182**, 795.
Pridham, J. B. (1960). In *Phenolics in Plants in Health and Disease*. Ed. by Pridham, J. B. London: Pergamon Press Ltd. (in the Press).

Biochem. J. (1960) 77, 564
Printed in Great Britain

The Reaction of Carbohydrases with Phenolic Glucosides

BY J. D. ANDERSON AND L. HOUGH
Department of Chemistry, The University, Bristol
AND J. B. PRIDHAM
Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 22 *March* 1960)

The biochemistry and physiology of phenolic glycosides in plants has been reviewed by Pridham (1960*a*). The biosynthesis of a phenolic diglucoside was first recorded by Miller (1941), who fed *o*-chlorophenol to *Gladiolus* corms and isolated the corresponding *o*-chlorophenyl β-gentiobioside. The enzymic formation of *p*-hydroxyphenyl β-gentiobioside from arbutin (*p*-hydroxyphenyl β-D-glucopyranoside) with a β-glucosidase preparation from aspen cambium (*Populus grandidentata*) has also been reported (Pridham, 1957, 1960*b*). With wheat-germ enzymes, however, the formation of this diglucoside from arbutin required uridine diphosphoglucose as a cofactor (Cardini & Yamaha, 1958, 1960). These wheat-germ extracts showed no β-glucosidase activity.

A study of the reaction of arbutin with enzyme preparations from almond (*Prunus amygdalus*) and broad bean (*Vicia faba* var. Johnson's Longpod) seeds suggests that β-glucosidase can catalyse the formation of a phenolic gentiobioside. When salicin (*o*-hydroxymethylphenyl β-D-glucopyranoside) was used as a substrate, *o*-hydroxybenzyl β-glucoside was produced, thus substantiating the early observations of Rabaté (1935), who investigated a similar reaction of salicin with an enzyme from willow (*Salix purpurea*) leaves.

MATERIALS AND METHODS

Ultraviolet-absorption spectra were measured in a Unicam SP. 500 spectrophotometer (1 cm. cell). Infrared measurements were made with a Unicam SP. 100 double-beam spectrophotometer. Control incubations were in all instances carried out with boiled enzyme preparations and all enzyme reactions were carried out in the presence of toluene.

Paper chromatography. Phenolic compounds and sugars were examined on Whatman no. 1 and no. 3 papers, respectively, by the descending technique. The solvent systems were: *A*, butan-1-ol–pyridine–water (6:4:3, by vol.); *B*, butan-1-ol–ethanol–water (40:11:19, by vol.) and *C*, ethyl acetate–acetic acid–water (9:2:2, by vol.). Diazotized *p*-nitroaniline–Na_2CO_3 (Swain, 1953) spray reagent was used to detect phenols and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), to detect the reducing sugars.

Paper electrophoresis. Whatman no. 3 paper and 0·1M-sodium borate buffer (pH 10·0) was used as described by Pridham (1959). Mobilities are expressed as M_{SA} values, i.e. relative to the mobility of salicylic acid.

p-*Hydroxyphenyl β-cellobioside.* This was synthesized by the method of Stánek & Kocourek (1953).

Enzyme preparations. Almond emulsin was prepared by the method described by Sumner & Somers (1947). Before use the enzyme solution was dialysed against running tap water (40 hr.) and then distilled water (5 hr.). Bean tissue was macerated with 0·07M-sodium phosphate (pH 7·0) buffer containing L-cysteine (0·5%, w/v) (about 1 g. of tissue/5 ml. of buffer) at 5°. The resulting extract was then centrifuged at 3000 rev./min. and the supernatant liquor dialysed against distilled water for 24 hr. at 5°. Enzyme preparations were made from (*a*) whole seeds (with testas removed) which had been soaked in water for 18 hr. at room temperature, (*b*) dormant embryos, (*c*) embryos from seeds treated as in (*a*), (*d*) embryos from germinating seeds which had been kept between layers of moist cotton wool for 6 days at room temperature, (*e*) cotyledons from seeds treated as in (*a*). Preparation (*a*) was fractionated by the addition of solid $(NH_4)_2SO_4$ to give 50% saturation, the mixture was then put into a separating funnel and the precipitated protein allowed to rise to the surface. The clear solution was run off and the suspended protein dialysed against distilled water (24 hr., 5°) and freeze-dried. Preparations (*c*) and (*e*) were both dialysed successively against three changes of distilled water (4 days), 0·05M-sodium acetate, pH 5 (24 hr.) and 0·07M-sodium phosphate, pH 8·5 (24 hr.). The resulting solutions were then adjusted to pH 6·5 with acetic acid.

Each enzyme preparation was incubated with 2·0% (w/v) solutions of salicin and arbutin for 18 hr. at 27° and the reaction mixtures were examined on paper chromatograms for sugars and phenols. The incubations with arbutin were repeated in the presence of D-glucono-1→4-lactone (0·7%, w/v) for the emulsin and the enzyme preparations (*a*) and (*b*).

Compound (I). Arbutin (3 g.) was incubated with emulsin solution (50 ml.) for 5 days at 37°; the incubation mixture was deproteinized by the method of Sevag, Lackman & Smollens (1938) and then extracted with ether to remove quinol. The aqueous solution was then passed through a small charcoal–Celite column (Whistler & Durso, 1950) which was washed with water to remove glucose. Aqueous ethanol (50%, v/v) was used to elute the glycosides from the column and the eluate was then concentrated under reduced pressure to a thin syrup. A phenolic compound (I) (30 mg.) was obtained from the latter by chromatographic fractionation on Whatman no. 3MM paper (solvent *B*). Complete hydrolysis of (I) was effected

by heating with N-H_2SO_4 (100°; 2 hr.) and also by treating with emulsin (27°, 18 hr.). Heating with N-HCl (100°, 10 min.) resulted in a partial hydrolysis of (I). Compound (I) (5 mg.) in water (1 ml.) was treated with bromine water (0·2M, 0·5 ml.) in the presence of Amberlite IR-4B (OH^- form) resin to neutralize any HBr formed. The solution was aerated to remove excess of Br_2 and then filtered and concentrated under reduced pressure to a syrup.

The optimum pH values for the formation of compound (I) with emulsin and with preparation (*a*) were estimated by incubating these enzymes (1 ml.) at 27° for 18 hr. with arbutin (5·0%, w/v) and either 0·1M-sodium citrate or 0·07M-sodium phosphate buffers (2 ml., pH range 3·0–8·0) and observing the rates at which compound (I) was formed, by paper chromatographic examination. Attempts to synthesize arbutin or compound (I), or both, were made by incubating 1 ml. of emulsin, preparation (*a*) or β-glucosidase (0·1%, w/v, aqueous solution; Sigma Chemical Co.) with quinol (20 mg.) and salicin, cellobiose, maltose or α-D-glucose 1-phosphate (100 mg. of each) in the presence of 0·05M-sodium acetate buffer (1 ml., pH 5·6) for 18 hr. at 27°.

Compound (II). Salicin (2 g.) was incubated with emulsin (66 ml.) for 7 days at 37°. The reaction mixture was then deproteinized and extracted with ether, as with compound (I), and the remaining solution concentrated and fractionated on Whatman no. 3MM paper (solvent *B*). Hydrolysis with N-H_2SO_4 and emulsin was carried out as with compound (I).

RESULTS

Paper-chromatographic examination of the products obtained by incubating arbutin with emulsin revealed the presence of glucose, quinol and a slower-moving component (I) (Table 1), which gave the same purple colour as arbutin with the diazonium spray reagent and also reacted with *p*-anisidine periodate (Bragg & Hough, 1958); small amounts of other compounds were also detected. In aqueous solution the spectrum of (I) resembled that of arbutin with an absorption maximum at 283 mμ (cf. Charlampowiczówna & Marchlewski, 1930). After addition of bromine water, this peak was replaced by two other maxima at 246 and 268 mμ which are characteristic of *p*-benzoquinone (cf. Braude, 1945). The solution after bromine treatment contained a compound which gave a brown colour with *p*-anisidine hydrochloride and co-chromatographed with authentic gentiobiose. (Gentiobiose was clearly distinguished from all other glucopyranosyl disaccharides by solvent *C*.) This evidence suggested that compound (I) was *p*-hydroxyphenyl gentiobioside. Complete acid hydrolysis of the gentiobioside yielded glucose and quinol and a partial hydrolysis gave a disaccharide which was chromatographically identical with gentiobiose. Treatment of the gentiobioside with emulsin produced glucose, quinol, and arbutin, thus suggesting the presence of β-links in the molecule; this was confirmed by infrared measurements on compound (I), which showed a characteristic absorption band at 891 $cm.^{-1}$ (Barker, Bourne, Stacey & Whiffen, 1954). The behaviour of the β-gentiobioside (I) differed from that of authentic *p*-hydroxyphenyl β-cellobioside on paper chromatography and electrophoresis (Table 1).

Incubation of arbutin with enzyme preparations from broad-bean tissues also produced a compound identical with *p*-hydroxyphenyl β-gentiobioside and the pH optimum for the formation of this compound was between 5 and 6 for both the bean [preparation (*a*)] and emulsin. All enzyme preparations which were tested produced the β-gentiobioside when incubated with arbutin. They also hydrolysed the latter compound, and salicin, to glucose and the corresponding phenolic aglycone. This reversible β-glucosidase activity was inhibited by D-glucono-1 → 4-lactone with emulsin and enzyme preparations (*b*) and (*a*). The protein fraction (*a*) obtained by precipitation with 50% ammonium sulphate saturation retained its ability to synthesize *p*-hydroxyphenyl β-gentiobioside as did preparations (*c*) and (*e*). Unsuccessful attempts were made to prepare arbutin and *p*-hydroxyphenyl β-gentiobioside by incubating emulsin [commercial and Sumner & Somers (1947) preparations] and bean enzymes with quinol and various glucose derivatives. *p*-Hydroxyphenyl β-gentiobioside was not produced when bean seeds or shoots were allowed to take up solutions of quinol (0·25%) or arbutin (0·5%) under the conditions of Pridham (1958) and Pridham & Saltmarsh (1960).

When the almond or bean enzyme preparations were incubated with salicin, the following were formed: glucose, saligenin (*o*-hydroxybenzyl alcohol) and a phenolic compound (II). Saligenin and (II) gave the same red colour with the

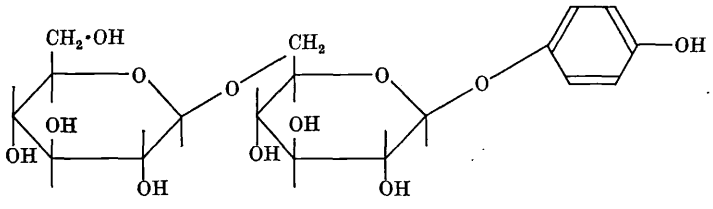
Table 1. *R_F and M_{SA} values of phenolic glucosides*

Glucoside	R_F in solvents			M_{SA} in 0·1M-sodium borate buffer (pH 10)
	A	*B*	*C*	
Arbutin	0·75	0·40	0·51	—
p-Hydroxyphenyl β-gentiobioside (I)	0·55	0·07	0·17	0·28
p-Hydroxyphenyl β-cellobioside	0·61	0·10	0·24	0·22
Salicin	—	0·56	0·48	0
o-Hydroxybenzyl β-glucoside (II)	—	0·59	0·48	0·15

diazonium reagent and both had similar u.v.-absorption spectra with maxima at 275 and 274 mμ, respectively (cf. Ramart-Lucas & Rabaté, 1933). In the presence of 0·002N-NaOH the absorption maximum of (II) shifted to 294 mμ. Salicin had approximately the same R_F value as (II) (Table 1) but did not react with the diazonium reagent. However, these two compounds were clearly separated by paper electrophoresis (Table 1). The absorption maximum of salicin at 268 mμ (cf. Charlampowiczówna & Marchlewski, 1930) was not affected by the addition of alkali. Glucose and saligenin only were detected in the acid and emulsin hydrolysates of (II).

DISCUSSION

The results obtained show that compound (I) is *p*-hydroxyphenyl β-gentiobioside, which was formed directly from arbutin. That the transfer of β-D-glucosyl units by β-glucosidase is involved with both the almond and bean preparations is suggested by the fact that β-glucopyranosides were hydrolysed by all of the enzyme solutions tested and that this activity and the ability to synthesize the gentiobioside was inhibited by D-glucono-1 → 4-lactone, a competitive inhibitor of β-glucosidase (Conchie & Levvy, 1955). In addition, the pH optima for β-glucosidase activity and the formation of the gentiobioside are similar and the synthesis of a 1 → 6 β-link by D-glucopyranosyl transfer involving β-glucosidase might be expected from the observations of Peat, Whelan & Hinson (1952) and Barker, Bourne, Hewitt & Stacey (1955).



(I)

The fact that extensively dialysed enzyme solutions and a protein fraction obtained by ammonium sulphate precipitation all synthesized the phenolic gentiobioside makes it unlikely that uridine diphosphoglucose participates in the reaction although the possibility that this nucleotide is present in a bound form cannot be ignored (cf. Buell, 1952).

The biosynthesis of phenolic diglucosides *in vivo* is more likely to involve uridine diphosphoglucose than a simple glucose donor, e.g. a disaccharide, in conjunction with β-glucosidase. It also seems probable that monoglucosylation of a phenolic hydroxyl group *in vivo* and *in vitro* requires the presence of a 'high-energy' glucose donor and that simple transglucosylation reactions are of little importance for this process (Pridham & Saltmarsh, 1960).

Compound (II) is an isomer of salicin with the β-glucopyranosyl group attached to the primary hydroxyl group of the saligenin nucleus. The u.v.-absorption measurements and paper electrophoresis indicated the presence of a free phenolic hydroxyl group. In this instance β-glucosidase would appear to have catalysed the transfer of a glucosyl radical from the phenolic to the primary hydroxyl group in the side chain with the formation of *o*-hydroxybenzyl β-glucopyranoside. This glycoside is also formed when glucose is incubated with saligenin in the presence of emulsin (Bourquelot & Hérissey, 1913), by the action of a willow-leaf extract on salicin (Rabaté, 1935) and by allowing broad-bean shoots and seeds to take up solutions of saligenin (Pridham, 1958; Pridham & Saltmarsh, 1960).

The possibility of glycoside synthesis should be borne in mind when carrying out kinetic studies on glycosidases, with phenolic or alcoholic glycosides as substrates, otherwise anomalous results may be obtained.

SUMMARY

1. The incubation of almond emulsin, or enzyme preparations from broad bean, with arbutin produces *p*-hydroxyphenyl β-gentiobioside.

2. A transglucosylation reaction catalysed by β-glucosidase is involved with arbutin molecules serving as both donors and acceptors of D-glucopyranosyl residues.

3. Salicin is partially converted into *o*-hydroxybenzyl β-glucopyranoside by the almond and broad-bean preparations.

The work described forms part of the programme of the Department of Scientific and Industrial Research.

REFERENCES

Barker, S. A., Bourne, E. J., Hewitt, G. C. & Stacey, M. (1955). *J. chem. Soc.* p. 3734.

Barker, S. A., Bourne, E. J., Stacey, M. & Whiffen, D. H. (1954). *J. chem. Soc.* p. 171.

Bourquelot, E. & Hérissey, H. (1913). *C.R. Acad. Sci., Paris*, **156**, 1790.
Bragg, P. D. & Hough, L. (1958). *J. chem. Soc.* p. 4050.
Braude, E. A. (1945). *J. chem. Soc.* p. 492.
Buell, M. V. (1952). *Fed. Proc.* **11**, 192.
Cardini, C. E. & Yamaha, T. (1958). *Nature, Lond.*, **182**, 1446.
Cardini, C. E. & Yamaha, T. (1960). *Arch. Biochem. Biophys.* **86**, 133.
Charlampowiczówna, B. & Marchlewski, L. (1930). *C.R. Soc. Polon. Phys.* p. 376.
Conchie, J. & Levvy, G. A. (1955). *Biochem. J.* **61**, xvi.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Miller, L. P. (1941). *Contr. Boyce Thompson Inst.* **12**, 163.
Peat, S., Whelan, W. J. & Hinson, K. A. (1952). *Nature, Lond.*, **170**, 1056.
Pridham, J. B. (1957). *Analyt. Chem.* **29**, 1167.
Pridham, J. B. (1958). *Nature, Lond.*, **182**, 795.
Pridham, J. B. (1959). *J. Chromat.* **2**, 605.
Pridham, J. B. (1960*a*). In *Phenolics in Plants in Health and Disease*. Ed. by Pridham, J. B. London: Pergamon Press Ltd. (in the Press).
Pridham, J. B. (1960*b*). *Biochem. J.* **76**, 13.
Pridham, J. B. & Saltmarsh, M. J. (1960). *Biochem. J.* **74**, 42P.
Rabaté, J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
Ramart-Lucas, P. & Rabaté, J. (1933). *C.R. Acad. Sci., Paris*, **196**, 1493.
Sevag, M. G., Lackman, D. B. & Smollens, J. (1938). *J. biol. Chem.* **124**, 425.
Stănek, J. & Kocourek, J. (1953). *Chem. Listy*, **47**, 697.
Sumner, J. B. & Somers, G. F. (1947). *Chemistry and Methods of Enzymes*, p. 96. New York: Academic Press Inc.
Swain, T. (1953). *Biochem. J.* **53**, 200.
Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.

REPRINTED FROM CHEMISTRY AND INDUSTRY, 1961, p. 1172

BIOSYNTHESIS OF PHENOLIC GLUCOSIDES IN THE ABSENCE OF UDPG*

By J. B. Pridham

Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey

Phenolic β-glucosides have been prepared *in vitro* by incubating various phenols with UDPG in the presence of plant protein preparations.[1,2] In addition, a β-glucopyranuronoside has been synthesised by the transfer of glucuronic acid from UDPGA† to quercetin using an enzyme from *Phaseolus vulgaris*.[3] Several attempts to glucosylate phenolic hydroxyl groups by transferase reactions involving "low energy" glucose donors have failed.[1,2,4] We are now able to report that glucose can be transferred from maltose to resorcinol using an *Aspergillus niger* preparation.

A crude aqueous extract of *A. niger* (Strain 152) was prepared as described by Barker and Fleetwood[5] and the pH then adjusted to 5·6 with sodium citrate buffer (final concentration 0·05 M). This solution, after dialysis against buffer at 5°, was observed to catalyse the synthesis of phenolic glucosides. Further purification of this enzyme was achieved by addition of ammonium sulphate to 40% saturation and solution of the precipitated protein in water followed by dialysis against water at 5°. Standard digests containing 0·1 M sodium acetate buffer (0·2 ml.), enzyme solution (0·2 ml.), phenol (0·1 mM) and glucose donor (0·1 mM) were used throughout these studies and incubations were carried out at 25° for 20 hr. in the presence of toluene. Chromatographic examination of a digest containing maltose and resorcinol revealed the presence of two phenolic compounds (A and B; Table) both giving the same pink colour as resorcinol β-glucoside with the diazotised *p*-nitroaniline-sodium hydroxide spray reagent.[6] Small amounts of compounds A and B were obtained by chromatographic resolution of a digest on Whatman No. 3 paper (butan-1-ol-ethanol-water (40:11:19, v/v) solvent). Compound A on hydrolysis with N-sulphuric acid (100°; 45 min.) yielded glucose and resorcinol. These products were also produced when compound A was treated with yeast α-glucosidase, honey invertase and the *A. niger* enzyme. The α-glucosidase and honey invertase preparations had little effect on resorcinol-β-glucoside. β-Glucosidase did not hydrolyse A even after incubation for 22 hr. at 35°. It seems probable therefore that compound A is resorcinol α-glucoside (3-hydroxyphenyl α-glucoside). The α-glucoside structure is also supported by comparing the paper chromatographic and electrophoretic behaviour of compound A with authentic resorcinol β-glucoside (Table).

*UDPG=uridine diphosphoglucose;
†UDPGA=uridine diphosphoglucuronic acid.

Table

Paper chromatographic and electrophoretic behaviour of compounds A and B and resorcinol β-glucoside

	A (*Resorcinol α-glucoside*)	*Resorcinol β-glucoside*	B (*Resorcinol α-iso-maltoside?*)
R_F values:			
Butan-1-ol-ethanol-water (40:11:19, v/v)	0·55	0·52	0·27
Ethyl acetate-acetic acid-water (9:2:2, v/v)	0·64	0·63	0·38
M_{SA} values:†			
0·1M-Sodium borate buffer (pH 10)	0·45	0·50	0·39

† Rate of movement relative to salicylic acid.[7]

Compound B was also hydrolysed rapidly by the *A. niger* enzyme to resorcinol and glucose; β-glucosidase was ineffective. Partial hydrolysis of B (N-H_2SO_4; 100°; 15 min.) produced a disaccharide which has been tentatively identified as isomaltose using paper chromatography and electrophoresis.

Preliminary experiments have shown that the *A. niger* enzyme can also transfer glucose from maltose to quinol, catechol and 1,2,4-trihydroxybenzene. With resorcinol as an acceptor, isomaltose also served as a glucose donor but glucoside synthesis was not apparent when D-glucose, α-D-glucose-1-phosphate, methyl α-D-glucoside, methyl β-D-glucoside, cellobiose, sucrose or UDPG were used as substrates.

Further studies with this enzyme are now in progress. The author wishes to thank Dr. W. J. Whelan for a gift of honey invertase.

Received May 30, 1961

References

1 Yamaha, T. & Cardini, C. E., *Arch. Biochem. Biophys.*, 1960, **86**, 127
2 Pridham, J. B. & Saltmarsh, M. J., *Biochem. J.*, 1960, **74**, 42P, and unpublished results
3 Marsh, C. A., *Biochim Biophys. Acta*, 1960, **44**, 359
4 Anderson, J. D., Hough, L. & Pridham, J. B., *Biochem. J.*, 1960, **77**, 564
5 Barker, S. A. & Fleetwood, J. G., *J. chem. Soc.*, 1957, 4857
6 Swain, T., *Biochem. J.*, 1953, **53**, 200
7 Pridham, J. B., *J. Chromatography*, 1959, **2**, 605

PRINTED BY RICHARD MADLEY LTD., 54 GRAFTON WAY, LONDON, W.1, ENGLAND

B7

Reprinted from *Phenolics in Plants in Health and Disease.*

PERGAMON PRESS

OXFORD · LONDON · NEW YORK · PARIS

Printed in Great Britain by Staples Printers Limited, Rochester

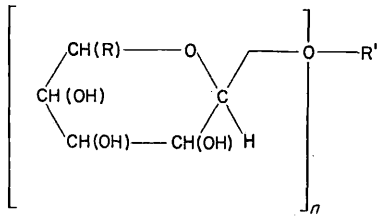
THE FORMATION AND POSSIBLE FUNCTION OF PHENOLIC GLYCOSIDES

J. B. PRIDHAM

Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey

PHENOLIC glycosides constitute a large and important group of naturally occurring compounds. In 1926 Bourquelot[1] detected glycosides in 205 out of the 281 species of phanerogams that he examined, and it is now generally recognized that the majority of low molecular weight phenols do occur as glycosides in plant tissues[2]. The highest concentrations of these compounds are reputed to be present in the cell sap of parenchymatous tissues and only small amounts are found in the xylem elements of woody plants[3].

The *O*-glycosides which will be examined in this review have the following general structure:



R = CH_2OH, H, CH_3 or CO_2H
R′ = Phenolic residue
n = 1–4.

Most attention will be paid to those compounds where R′ is a phenolic residue although it will be necessary to consider some other derivatives.

The carbohydrate moieties of the naturally occurring phenolic glycosides may consist of single or short chains of monosaccharide residues. In the case of the single residue the aglycone is bonded to the glycosidic carbon atom of the sugar and, with few exceptions, the linkage is β and the sugar is in the pyranose ring form. Where several monosaccharide residues are present these may occur as non-reducing linear chains, or more than one hydroxyl group of the aglycone can be glycosylated. D-Glucosides are the most common naturally occurring plant glycosides. Other monosaccharides occurring as glycosides include D-galactose, D-mannose, L-rhamnose, D-fucose, D-glucuronic acid, D-xylose and L-arabinose[4]. Disaccharide glycosides have been

reviewed by McIlroy[4] and Pigman[5]. Phenolic derivatives of primverose (6-*O*-β-D-xylopyranosyl-D-glucopyranose), rutinose (6-*O*-β-L-rhamnopyranosyl-D-glucopyranose) and vicianose (6-*O*-β-L-arabopyranosyl-D-glucopyranose) are well-known examples and in the case of the alkaloid glycosides tri- and tetra-saccharides have been characterized[6].

Chains of monosaccharide residues are easily degraded by chemical and enzymic hydrolyses, so great care should be taken during the isolation of plant glycosides to control the pH of the extracts and to inactivate all glycosidases. Such precautions have not always been observed and many reported monosaccharide constituents may have been higher saccharides.

A great deal is known about the biosynthesis of carbohydrates[7] and phenolic compounds[8] but until very recent times the mechanism of the formation of glycosidic derivatives of phenols was not known, although it was generally agreed that the sugar moiety was attached at a late stage in the formation of the glycoside. In 1913 Bourquelot and his collaborators[9-12] reported the formation of glycosides when high concentrations of alcohols were incubated with monosaccharides in the presence of almond emulsin or yeast enzymes. The first biosynthesis of phenolic glycosides, however, was reported by Ciamician and Ravenna[13] who treated maize seedlings with phenols and obtained glucosides. It is interesting to note that the latter workers isolated salicin [(*o*-hydroxymethylphenyl)-β-glucoside] from maize which had been treated with saligenin (*o*-hydroxybenzyl alcohol) whereas Bourquelot[11] obtained *o*-hydroxybenzyl)-β-glucoside when saligenin was incubated with glucose and emulsin. Miwa *et al*[14] infused leaf discs with glucose together with various phenolic compounds. The β-glucosides of vanillin, salicylaldehyde and quinol were obtained in this way and increased yields of glucosides were obtained by using glucose-1-phosphate instead of glucose. Grinding the leaves caused loss of synthetic activity, but cell-free enzyme preparations were obtained from broad bean (*Vicia faba*) and blue pine (*Pinus sativa*) seeds. Dialysis of these preparations caused inactivation but activity was restored by the addition of "yeast nucleotide" and adenosine triphosphate. Later work by Hutchinson *et al.*[15] has substantiated many of these observations. These workers were chiefly concerned with the formation of phlorin (phloroglucinol-β-glucoside) by apple leaf discs, which had been infiltrated with phloroglucinol and ^{14}C-glucose. Infiltration of leaves from a number of other plant species was also studied using phloroglucinol and other phenols. In all cases the corresponding glucosides were detected in the tissues. Pridham[16] has recently studied the uptake of aqueous solutions at low molecular weight phenols by broad bean shoots. Catechol, quinol, resorcinol and phloroglucinol were all converted to mono-β-glucosides by the plant, and hydroxyquinol and pyrogallol were also glucosylated but with the latter two compounds the number and position of the glucose residues

has so far not been determined. Phenol was found to be highly toxic to the bean and glucoside formation could not be demonstrated.* (*o*-Hydroxybenzyl)-β-glucoside, and not salicin, was produced when saligenin was fed to bean shoots. Similar results have been obtained by allowing bean seeds to germinate between layers of cotton wool soaked in solutions of the phenols[18]. Increased yields of glucosides were obtained in this way.

The glucose donor for glucoside synthesis *in vivo* is probably uridine diphosphate glucose (UDPG), a nucleotide which presumably occurs widely in plant tissues[19, 20]. Cardini and his co-workers[21, 22] have been able to synthesize glucosides by incubating phenols with UDPG in the presence of an enzyme preparation from wheat germ. The use of other potential glucose donors such as glucose-l-phosphate, sucrose, cellobiose and maltose was also investigated but in all cases no synthetic activity was observed. Enzyme preparations from broad bean seeds will also catalyse the glucosylation of phenols by UDPG[18]. Uridine diphosphate derivatives of galactose, xylose, arabinose[20] and glucuronic acid[23] have been isolated from plant tissues and it is probable that these compounds take part in the formation of glycosides possessing the corresponding sugar residues.

The biosynthesis of a phenolic diglycoside was first reported by Miller[24, 25] who treated gladioli corms and tomato plants with *o*-chlorophenol and isolated *o*-chlorophenyl-β-gentiobioside. Miller also obtained 2 : 2 : 2-trichloroethyl-β-gentiobioside by treating various plant species with chloral hydrate[25-27]. (*p*-Hydroxyphenyl)-β-gentiobioside has been synthesized by incubating arbutin [(*p*-hydroxyphenyl)-β-glucoside] with dialysed protein preparations from the inner bark of trembling aspen (*Populus tremuloides*)[28] and from sweet almond and broad bean seeds[29]. In the case of the latter preparation activity was still present after extensive dialysis against water and acidic (pH 5·0) and alkaline (pH 8·5) buffers, and the β-glucosidase and synthetic activities of the preparation were both strongly inhibited by D-glucono-1→4-lactone[30]. Incubation of the protein solution with quinol and glucose, or quinol and gentiobiose, produced no gentiobioside or arbutin. The evidence suggests that the mechanism involved is a transglucosylation catalysed by β-glucosidase with arbutin molecules, acting as both glucosyl donors and acceptors, and that uridine coenzymes are not involved (see Fig. 1). Cardini and Yamaha[22] detected (*p*-hydroxyphenyl)-β-gentiobioside, in addition to arbutin, when quinol was incubated with UDPG in the presence of a wheat germ extract. The amount of gentiobioside formed was increased when arbutin was substituted for quinol and synthesis did not occur when UDPG was replaced by other potential glucose donors. β-Glucosidase activity was not apparent in the wheat germ preparations and recent work with ^{14}C-UDPG has proved beyond doubt that this nucleotide

* Nystrom *et al.*[17] have recently demonstrated that phenyl-β-glucoside is formed when barley or wheat leaves are fed with traces of phenol.

is the glucose donor for glucoside and gentiobioside formation in wheat germ extracts[31].

There would appear to be two mechanisms for the biosynthesis of phenolic diglucosides but *in vivo* the pathway involving the uridine coenzyme is probably the more important.

The reported functions of phenolic glycosides in plant tissues are mostly hypothetical. Pfeffer[32] stated that glycosides formed sugar reserves and this was to some extent supported by the work of Bridel[33] who showed that glucosides disappeared during the germination of seeds from annual plants.

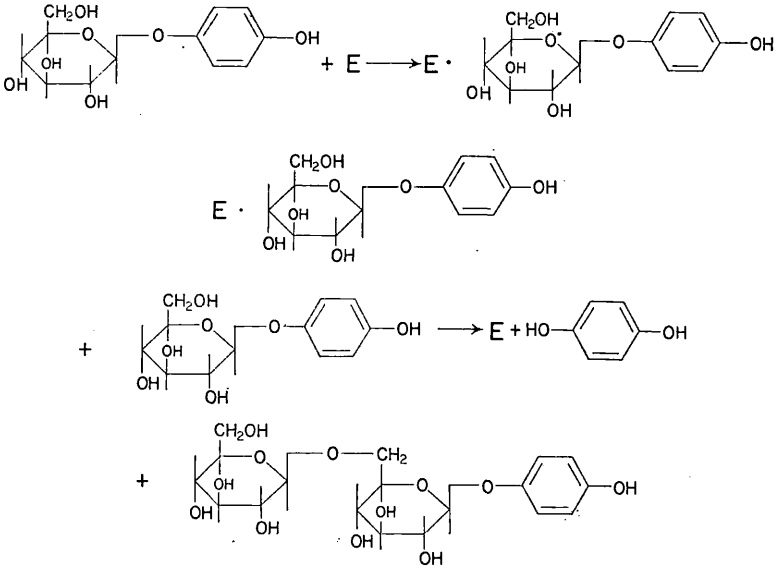


FIG. 1. Suggested mechanism for the formation of (*p*-hydroxyphenyl)-β-gentiobioside. (E=β-glucosidase.)

Weevers[34] observed a decrease in the salicin content of willow during budding and fruiting, but Wasicky[35] suggested that the fluctuation of the glycoside contents of plant tissues was associated with osmoregulation rather than a metabolism of reserves.

Glycosides, according to Straub[36] are by-products which are not reintroduced into the metabolism. It is difficult to believe, however, that these compounds are merely waste products particularly in view of the many structurally different glycosidic residues which are known to occur. For example, galactose, glucose, arabinose, xylose, rhamnose and rutinose derivatives of quercetin are found in apple skin[37].

At the present time the role of glycosides as carbohydrate reserves is not clear. Under normal conditions glucose is readily available to the plant and glucosides are therefore probably not important sources of this sugar. They

may, of course, be important under conditions of starvation. In some plant species glycosides could form soluble reserves of the less common sugars, such as L-rhamnose and L-arabinose.

The variety and complexity of the oligoglycosides suggests that these compounds are biochemically important although, here again, there is little experimental evidence to support this supposition. The biological reactivity of the aglycone in the plant may perhaps be determined by the glycosidic group, the latter only allowing interaction with specific enzymes. In the case of the strophanthidin glycosides the degree of toxicity to animals is determined by the nature of the sugar residue[38].

It is tempting to suggest that oligoglycosides can function as substrates for the biosynthesis of complex plant polysaccharides (heteroglycans). The biosynthesis of homoglycans, such as amylose, is now well understood[39] but the mechanism of the formation of highly branched heteroglycans, such as the plant gums, remains obscure. These molecules may be synthesized from oligosaccharide "building-blocks" and not by a gradual build-up from single primer units, which because of steric effects might be difficult. Such oligosaccharides, however, have not been detected in a free state in plant tissues but they could conceivably be preformed as derivatives of phenols (or nucleotides) and then incorporated *in toto* into the heteroglycan by transglycosylation. In this connexion it is interesting to note that disaccharidases from *Rhamnus sp.* have been described[40]. These enzymes are reported to hydrolyse primverosides and rutinosides into aglycone and disaccharide. A reversal of this type of reaction could play a role in the biosynthesis of heteroglycans by the pathway described.

One valid function of glycoside formation would appear to be detoxification of compounds which are harmful to the plant[4, 15, 16]. An analogous reaction in the animal body is glucuronoside formation. Many phenolic compounds are phytotoxic. They are usually lipophilic in character but possess hydrophilic groups and are therefore surface active. Such compounds could interfere with the function of cell vacuoles, by interaction with the tonoplast membrane[41]. Some phenols also interfere with oxidative phosphorylation[42] and the related phenomenon of mitosis[43].

The toxic effects of some phenols can be greatly reduced by glycosylation of one or more hydroxyl groups. This can readily be demonstrated by comparing the germination of broad bean seeds on cotton wool soaked in equimolar solutions of quinol and arbutin. It can be shown that both compounds readily enter the tissues and with arbutin germination proceeds normally but with quinol the seeds rapidly blacken and die[18].

A detoxification mechanism is no doubt essential for the plant in order that phenols and other harmful compounds which enter the tissues may be counteracted. These compounds could conceivably be derived from three different sources: (a) normal plant metabolism, (b) small amounts absorbed

from the soil, (c) externally applied compounds, such as fungicides and growth regulators. With regard to the latter, Towers *et al.* [44] have shown that maleic hydrazide (a compound with phenolic properties) is converted to a glucoside by plant tissues and they suggest that this is the reason why relatively high concentrations of the reagent are needed for effective growth inhibition.

The solubilization of phenolic compounds is possibly another important aspect of glycoside biosynthesis. Many naturally occurring phenols are relatively insoluble in aqueous solutions at physiological pH values and glycosylation might therefore be necessary to allow translocation. An increase in hydrophilic properties would also prevent phenolic compounds from interfering with the tonoplast membrane[41]. A further possible function of glycoside formation could be the stabilization of labile compounds[4] and in particular, phenols which are prone to oxidation.

Tannins and other phenolic compounds, such as chlorogenic acid, are reputed to be important factors in resistance to disease[45, 46]. Some plant glycosides may also be latent antimicrobic substances. Invasion of the tissues, by micro-organisms, would release glycosidases by breakdown of the plant cells and these enzymes could then liberate phenols or other toxic compounds to combat the infection.

Our knowledge of the true role of glycosides in plant tissues is at present negligible, but it is hoped that future studies using isotopic tracers and modern analytical techniques will either prove or disprove some of the hypotheses that have been made. Further work on the structure and distribution of glycosides with complex carbohydrate moieties would be of particular interest and might reveal important aspects of plant metabolism.

REFERENCES

1. BOURQUELOT E. quoted by BEQUIN C. (1926) *Pharm. Acta. Helv.*, **1**, 90.
2. BARUAH P. and SWAIN T. (1957) *Biochem. J.* **66**, 321.
3. WISE L. E. and JAHN E. C. (1952) *Wood Chemistry*, Vol. I, p. 648. Reinhold Publ. Corp., New York.
4. MCILROY R. J. (1951) *The Plant Glycosides.* Edward Arnold, London.
5. PIGMAN W. (1957) *The Carbohydrates.* Academic Press Inc., New York.
6. KUHN R., LOW I. and TRISCHMANN H. (1955) *Ber. Dtsch. Chem. Ges.*, **88**, 1492; **88**, 1670; (1957) **90**, 203.
7. HOUGH L. and JONES J. K. N. (1956) *Advanc. Carbohydrate Chem.*, **11**, 185.
8. BOGORAD L (1958) *Ann. Rev. Plant Physiol.*, **9**, 417.
   BIRCH A. J. and SMITH H. (1958) *Chem. Soc. Special Publ.*, No. 12, p. 1.
   EHRENSVÄRD G. (1958) *Chem. Soc. Special Publ.*, No. 12, p. 17.
9. BOURQUELOT E., HÉRISSEY H. and BRIDEL M. (1913) *Compt. rend.*, **156**, 168, 330, 491, 1493.
10. BOURQUELOT E. and BRIDEL M. (1913) *Compt. rend.*, **156**, 827, 1104.
11. BOURQUELOT E. and HÉRISSEY H. (1913) *Compt. rend.*, **156**, 1790.
12. BOURQUELOT E. and VERDON E. (1913) *Compt. rend.*, **156**, 957, 1264, 1638.
13. CIAMICIAN G. and RAVENNA C. (1916) *Atti. reale accad. Lincei*, **25**, 3.
14. MIWA T., NAKAMURA S. and SHIBATA A. (1957) *Koso Kagaku Shinpojiumu*, **12**, 48.
15. HUTCHINSON A., ROY C. and TOWERS G. H. N. (1958) *Nature, Lond.*, **181**, 841.

16. PRIDHAM J. B. (1958) *Nature, Lond.*, **182,** 795.
17. NYSTROM C. W., TOLBERT N. E. and WENDER S. H. (1959) *Plant Physiol.*, **34,** 142.
18. PRIDHAM J. B. and SALTMARSH M. J. (1960) *Biochem. J.*, **74,** *42p.*
19. BURMA D. P. and MORTIMER D. C. (1956) *Arch. Biochem. Biophys.*, **62,** 16.
20. GINSBURGH V., STUMPF P. K. and HASSID W. Z. (1956) *J. Biol. Chem.*, **223,** 977.
21. CARDINI C. E. and LELOIR L. F. (1957) *Ciencia e invest.*, **13,** 514.
22. CARDINI C. E. and YAMAHA T. (1958) *Nature, Lond.*, **182,** 1446.
23. SOLMS J., FEINGOLD D. S. and HASSID W. Z. (1957) *J. Amer. chem. Soc.*, **79,** 2342.
24. MILLER L. P. (1940) *Science* **92,** 42.
25. MILLER L. P. (1941) *Contribs. Boyce Thompson Inst.*, **12,** 163.
26. MILLER L. P. (1941) *Contribs. Boyce Thompson Inst.*, **12,** 15.
27. MILLER L. P. (1943) *Contribs. Boyce Thompson Inst.*, **13,** 185.
28. PRIDHAM J. B. (1957) *Anal. Chem.*, **29,** 1167.
29. ANDERSON D., HOUGH L. and PRIDHAM J. B. Unpublished results.
30. CONCHIE J. and LEVVY G. K. (1955) *Biochem. J.*, **61,** XVI.
31. CARDINI C. E. (1958) Personal communication.
32. PFEFFER W. (1897) *Pflanzen physiol.*, **1,** 492.
33. BRIDEL M. (1926) *Rev. gén. Sci. pur. appl.*, **37,** 134.
34. WEEVERS T. (1904) *Jb. wiss Bot.*, **39,** 229.
35. WASICKY R. (1921) *Biochem. Z.*, **113,** 1.
36. STRAUB W. (1917) *Biochem. Z.*, **82,** 48.
37. SIEGELMAN H. W. (1955) *J. Biol. Chem.*, **213,** 647.
38. UHLE F. C. and ELDERFIELD R. C. (1943) *J. Org. Chem.*, **8,** 162.
39. BARKER S. A. and BOURNE E. J. (1953) *Quart. Revs.*, **7,** 56.
40. CHARAUX C. R. (1924) *Compt. rend.*, **178,** 1312.
BRIDEL M. (1924) *Compt. rend.*, **179,** 780.
41. FREY-WYSSLING A. (1942) *Naturwiss.*, **33,** 500.
42. BONNER J. (1949) *Ann. J. Bot.*, **36,** 429.
MILLERD A., BONNER J. and BIALE J. B. (1953) *Plant Physiol.*, **28,** 521.
43. DEYSON G. and TRUHART R. (1957) *Compt. rend. soc. biol.*, **151,** 1678, 1719.
44. TOWERS G. H. N. and HUTCHINSON A. (1958) *Nature, Lond.*, **181,** 1535.
45. THATCHER R. W. (1921) *Chemistry of Plant Life*, p. 99, McGraw Hill, New York.
GAUMANN E. (1950) *Principles of Plant Infection*, p. 273. (Crosby Lockwood, London).
46. KUĆ J., HENZE R. E., ULLSTRUP A. J. and QUACKENBUSH F. W. (1956) *J. Amer. chem. Soc.*, **78,** 3123.
CRUICKSHANK I. A. M. and SWAIN T. (1956) *J. Exptl. Bot.*, **7,** 410.

DISCUSSION

Dr. E. C. BATE-SMITH asked if the given list of sugar residues found in glycosides was exhaustive or whether it was representative of a wider range.

Dr. J. B. PRIDHAM said that the list was a representative one and included only common sugars found in combination.

Dr. J. B. HARBORNE stated that arabinose was rarely found in glycosidic combination with phenolics although it was very common in plant polysaccharides and asked whether Dr. Pridham had any observation to make regarding this.

Dr. J. B. PRIDHAM agreed that at present arabinose had not been commonly found in phenolic glycosides but suggested that many extraction procedures which had been used would hydrolyse off these sugar residues particularly if they were in the furanose form. He felt that phenolic glycosides could act as readily transportable sources of rarer sugars for polysaccharide synthesis.

[*Reprinted from the Proceedings of the Biochemical Society*, 11-12 *January* 1962.
Biochem. J., 1962, Vol. 82, No. 3, 44-45 P.]

The Formation of Acidic Derivatives of Phenolic Glucosides by *Vicia faba* Seeds

By J. B. PRIDHAM and MARGARET J. SALTMARSH.
(*Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey*)

Plants readily convert foreign phenols into the corresponding β-D-glucosides probably through the agency of uridine diphosphate glucose (Pridham, 1961). Careful examination of extracts of *V. faba* seeds which had been treated with saligenin, quinol, catechol and resorcinol showed that, in each case, small amounts of acidic sulphur-containing materials had been produced in addition to the glucosides.

Chromatographic fractionation of a methanolic extract of bean seeds which had been fed with resorcinol produced a compound X in low yield. (The compound was labile and in aqueous or ethanolic solution decomposed forming resorcinol β-glucoside.) On paper chromatograms and electrophoretograms it behaved as an acidic compound and hydrolysis with 0·5N-NaOH (70°) rapidly produced resorcinol β-glucoside and sulphate ions. Acidic hydrolysis of X yielded glucose and resorcinol and methylation (Kuhn, Trischmann & Löw, 1955) followed by alkaline and acidic hydrolyses gave resorcinol monomethyl ether and 2,3,4-tri-*O*-methyl-D-glucose. A glycosulphatase preparation and β-glucosidase both showed negligible activity towards X.

The experimental evidence in the main suggests that X is the 6′-sulphate of resorcinol β-glucoside but some of its properties, and in particular the action of alkali, are not characteristic of other glycoside 6-sulphates (Percival, 1949). Studies with 6′-tosylarbutin have to some extent explained the apparent anomolous behaviour with alkali and the possible structure for X and the other acidic derivatives were discussed with reference to this model compound.

The authors acknowledge the interest shown by Professor E. J. Bourne and Dr D. A. Rees and thank Dr A. G. Lloyd for a glycosulphatase preparation. One of us (M.J.S.) is indebted to the D.S.I.R. for a grant.

Kuhn, R., Trischmann, H. & Löw, I. (1955). *Angew. Chem.* **67**, 32.

Percival, E. G. V. (1949). *Quart. Rev.* **3**, 369.

Pridham, J. B. (1961). In *Phenolics in Plants in Health and Disease*, p. 9. Ed. by Pridham, J. B. London: Pergamon Press.

B9

Reprinted from

PHYTOCHEMISTRY

An International Journal of Plant Biochemistry

PERGAMON PRESS

OXFORD · LONDON · NEW YORK · PARIS

Phytochemistry, 1963, Vol. 2, pp. 225 to 230. Pergamon Press Ltd. Printed in England

THE IDENTIFICATION OF NATURALLY OCCURRING CINNAMIC ACID DERIVATIVES

E. J. BOURNE, NORMA J. MACLEOD and J. B. PRIDHAM
Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey

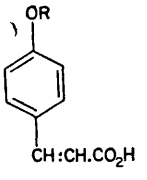
(*Received* 18 *January* 1963)

Abstract—Boron trichloride demethylates and deesterifies naturally occurring methoxy and hydroxy cinnamic acid derivatives. In the presence of methanol, the halide produces the corresponding methyl cinnamate. The procedure, in conjunction with paper chromatography and electrophoresis, may be used to classify cinnamic acids in plant tissues into one or more of three groups.

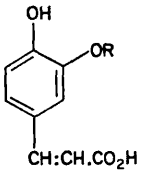
INTRODUCTION

IN RECENT years much attention has been focussed on the derivatives of cinnamic acid which occur in higher plants. These compounds may play a role in disease resistance,[1] they are plant growth factors[2] and are probably important intermediates in the biosynthesis of flavonoids and lignin.[3] From a botanical point of view, these derivatives also have taxonomic significance.[4]

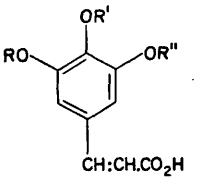
The most commonly occurring cinnamic acids are *p*-coumaric acid (I, R=H), caffeic acid (II, R=H), ferulic acid (II, $R=CH_3$) and sinapic acid (III, R′=H, $R=R''=CH_3$).



I



II



III

In addition *p*-methoxycinnamic acid (I, $R=CH_3$) and 3,4,5-trimethoxycinnamic acid (III, $R=R'=R''=CH_3$) have recently been reported,[5] and an ester of cinnamic acid itself is formed when phenylalanine is fed to potatoes.[6]

In rare instances, the cinnamic acids occur in the free state in plant tissues (e.g. Asen and Emsweller[7]) but they are more usually present in combination with sugars or quinic

[1] H. R. ANGEL, J. C. WALKER and K. D. LINK, *Phytopathology* **20**, 431 (1930).
G. JOHNSON and L. A. SCHAAL, *Science* **115**, 627 (1952).
I. A. M. CRUICKSHANK and T. SWAIN, *J. Exp. Botany* **7**, 410 (1956).
J. KUC, R. E. HENZE, A. J. ULLSTRUP and F. QUACKENBUSH, *J. Am. Chem. Soc.* **78**, 3123 (1956).
A. E. FLOOD and D. S. KIRKHAM in *Phenolics in Plants in Health and Disease* (ed. J. B. PRIDHAM) p. 81, Pergamon Press, London (1960).
A. C. HULME and K. L. EDNEY in *Phenolics in Plants in Health and Disease* (ed. J. B. PRIDHAM) p. 87, Pergamon Press, London (1960).

[2] J. DE ROUBAIX and O. LAZAR in *Phenolics in Plants in Health and Disease* (ed. J. B. PRIDHAM) p. 35, Pergamon Press, London (1960).
C. F. VAN SUMERE in *Phenolics in Plants in Health and Disease* (ed. J. B. PRIDHAM) p. 25, Pergamon Press, London (1960).

[3] A. C. NEISH, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).

[4] E. C. BATE-SMITH, *Sci. Proc. Roy. Dublin Soc.* **27**, 165 (1956).

[5] J. J. CORNER, J. B. HARBORNE, S. G. HUMPHRIES and W. D. OLLIS, *Phytochemistry* **1**, 73 (1962).

[6] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).

[7] S. ASEN and S. L. EMSWELLER, *Phytochemistry* **1**, 169 (1962).

acid. Thus the carboxyl groups are often esterified with glucose or quinic acid and occasionally with disaccharides. In addition, the phenolic hydroxyl groups can also be glucosylated.[8]

These derivatives normally occur in low concentrations in the tissues and consequently are best identified by methods such as paper chromatography and electrophoresis and spectroscopy. Degradation of the ester derivatives can be achieved by hydrolysis with acid or alkali and by the use of enzymes such as β-glucosidase and anthocyanase. The phenolic glycosides can be hydrolysed with acid or glycosidases.[8]

In view of the facile cleavage of ethers,[9] esters[10] and glycosides[11] to the parent hydroxy compounds by boron trichloride a successful attempt has been made to convert naturally occurring cinnamic acid derivatives to the corresponding mono-, di- or tri-hydroxy cinnamic acids (isolated as methyl esters) with the aid of this reagent.

RESULTS AND DISCUSSION

Preliminary studies were designed to examine the effect of the reagent on cinnamic, *p*-coumaric, caffeic and sinapic acids. These compounds were treated with boron trichloride in methylene chloride for 2 hr at −78° followed by 20 hr at room temperature. Excess halide was removed by distillation under reduced pressure and the reaction products were then exhaustively distilled with methanol to remove borate.

Cinnamic acid itself under these conditions yielded a crystalline compound (IV) which gave the correct analysis for methyl cinnamate. The structure was confirmed by (i) hydrolysis, which produced cinnamic acid and methanol, (ii) the preparation of a dibromide derivative and (iii) comparative ultraviolet and infrared spectrophotometry.

Similarly, *p*-coumaric, caffeic and sinapic acids were converted by boron trichloride followed by methanol to methyl *p*-coumarate (V), methyl caffeate (VI) and methyl 3,4,5-trihydroxycinnamate (VII), respectively.

The paper chromatographic and electrophoretic properties of the three methyl hydroxycinnamates have been studied in some detail and compared with those of the parent cinnamic acids (Table 1). The best procedure for the resolution of mixtures of these compounds is paper chromatography using ethyl acetate–acetic acid–water solvent and molybdate-treated papers. This system clearly separates the three esters and has the added advantage that the molybdate complexes of the di- and tri-hydroxy derivatives are clearly discernible as brown spots on chromatograms. Paper electrophoresis, using borate and molybdate buffers, is also a useful procedure for separation and identification. On paper, the three esters exhibit characteristic fluorescences with ammonia under ultraviolet light and can also be located with diazotised *p*-nitroaniline.

The reaction of boron trichloride and methanol with ferulic, 3,4-dimethoxycinnamic and chlorogenic acids was also examined in small scale experiments. All these compounds gave rise to methyl caffeate (VI), which was identified in each case by paper chromatography and electrophoresis.

The method has been applied to plant material containing cinnamic acid derivatives. Thus, powdered coffee beans, which contains chlorogenic acid,[12] gave rise to (VI) and compounds (V) and (VI) were produced by treating a crude extract of *Antirrhinum majus*

[8] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).
[9] W. GERRARD, *The Organic Chemistry of Boron*, Academic Press, London (1961).
[10] W. GERRARD and M. A. WHEELANS, *J. Chem. Soc.* 4296 (1956).
[11] T. G. BONNER, E. J. BOURNE and S. MCNALLY, *J. Chem. Soc.* 2929 (1960).
[12] R. G. MOORES, D. L. MCDERMOTT and T. R. WOOD, *Anal. Chem.* **20**, 620 (1948).

TABLE 1. CHROMATOGRAPHIC AND ELECTROPHORETIC PROPERTIES AND COLOUR REACTIONS OF CINNAMIC ACIDS AND METHYL CINNAMATES

Compound	R_f values in:			
	BEW		EAW	
	Plain paper	Borate-treated paper	Plain paper	Molybdate-treated paper
p-Coumaric acid	0·89	0·36	0·96	0·96
Caffeic acid	0·71	0·03	0·89	0·29
Sinapic acid	0·86	0·28	0·90	0·89
Methyl *p*-Coumarate	0·94	0·96	0·97	0·96
Methyl caffeate	0·86	0·61	0·92	0·68
Methyl 3,4,5-trihydroxycinnamate*	0·80	0·48	0·87	0·34

Compound	M_{SA} values† in:			Appearance in U.V. light and NH_3	Colour with diazotised *p*-nitroaniline +NaOH
	Borate	Molybdate	Acetate		
p-Coumaric acid	0·98	0·44	0·35	Violet	Mauve
Caffeic acid	1·04	0·72	0·20	Light blue	Yellow-brown
Sinapic acid	0·64	0·28	0·21	Green	Red
Methyl *p*-Coumarate	0·38	0	0	Violet	Mauve
Methyl caffeate	0·45	0·78	0	Light blue	Yellow-brown
Methyl 3,4,5-trihydroxycinnamate*	0·61	0·82	0	Blue	Brown

* This compound tends to streak on paper chromatograms and electrophoretograms.
† Mobility relative to that of salicylic acid.

flowers with boron trichloride and methanol. These flowers contain glucose esters of *p*-coumaric, caffeic and ferulic acids.[8] The action of boron trichloride on *O*-glucosides of cinnamic acids has not been investigated but there is no doubt that these would also be cleaved by the reagent (cf. Bonner, Bourne and McNally[11]).

The procedure described is an additional aid to the identification of cinnamic acids in plants. The method classifies those compounds present in the tissues into one or more of three hydroxycinnamic acid "families" and it is extremely simple to carry out.

EXPERIMENTAL

Paper Chromatography

The solvents used were: BEW (butan-1-ol, ethanol, water 40 : 11 : 19, v/v), EAW (ethyl acetate, acetic acid, water 9 : 2 : 2, v/v). Whatman No. 3 paper was used; impregnation with molybdate and borate was achieved by dipping papers into 0·1M sodium molybdate and 0·1M sodium borate solutions, respectively, blotting and air-drying.

Paper Electrophoresis

This was carried out, as described by Pridham,[13] with the following electrolyte solutions: 0·2M sodium borate (pH 10·0), $8{\cdot}1 \times 10^{-3}$M ammonium molybdate (pH 5·2) and 0·2M sodium acetate (pH 5·2).

[13] J. B. PRIDHAM, *J. Chromatog.* **2**, 605 (1959).

Location of Compounds

Chromatograms and electrophoretograms were viewed under ultraviolet light ("Chromatolite", Hanovia Ltd., Slough, Bucks) after exposure to ammonia vapour. They were also sprayed with diazotised *p*-nitroaniline followed by N NaOH solution.[14]

Absorption Spectra

Ultraviolet spectra were measured with a Unicam SP.500 spectrophotometer using 1 cm silica cells. Infrared measurements were made with a Perkin–Elmer (model No. 137) spectrophotometer.

Standard Conditions for Reaction with Boron Trichloride

The dried cinnamic acid was dissolved or suspended in methylene chloride and placed in a flask fitted with a silica gel trap. The flask was cooled to −78° and boron trichloride (12 ml) at the same temperature added. After 2 hr at −78° the reaction mixture was allowed to stand for approximately 20 hr at room temperature. Excess boron trichloride was then removed under reduced pressure and the borate ions removed by repeated distillation with dry methanol. The mixture was then concentrated to dryness under reduced pressure.

Reaction with cinnamic acid

Cinnamic acid (4·3 g) in methylene chloride (80 ml) under the above conditions yielded a residue which was distilled in a Jackson unit at 0·1 mm pressure. A pale yellow oil came over at 68–72° and on cooling, crystals (IV, 2·9 g) m.p. 35°, were produced. The compound (IV) gave a positive ester test with ferric chloride and hydroxylamine and had ultraviolet and infrared spectra identical to those of methyl cinnamate (lit. m.p. 34·7°[15]), and a molecular weight determination (166; determined cryoscopically in benzene) confirmed this. (Found: C, 74·5; H, 6·3; OMe, 19·1. Calc. for $C_{10}H_{10}O_2$: C, 74·1; H, 6·2; OMe, 19·1%.)

Compound (IV) (0·1 g) was heated on a boiling water bath for 2 hr with 0·025N NaOH (30 ml). The hydrolysate was then distilled and the methanol, which came over in the distillate, was characterized by the preparation of the 3,5-dinitrobenzoyl derivative (m.p. 108°; lit. m.p. 108°[16]). The residue remaining after distillation was dissolved in water and acidified with 2N H_2SO_4. Cinnamic acid (m.p. and mixed m.p. 133°) crystallized out.

Treatment of (IV) (0·15 g) with bromine in carbon tetrachloride followed by concentration of the reaction mixture yielded crystalline methyl α,β-dibromodihydrocinnamate (0·08 g) which on recrystallization from light petroleum (b.p. 60–80°) had m.p. 117° (lit. m.p. 117°[17]). (Found: C, 37·8; H, 3·5; Br, 48·8. Calc. for $C_{10}H_{10}O_2Br_2$: C, 37·3; H, 3·1; Br, 49·6%).

Reaction with p-*Coumaric Acid*

p-Coumaric acid (4·9 g) in methylene chloride (80 ml), when treated with boron trichloride and methanol, yielded a compound (V), which was removed from the reaction mixture by distillation at 75–80° (0·1 mm). The resulting oil, on cooling, yielded crystals (3·65 g, m.p. 137°). (Found: C, 67·3; H, 5·6. Calc. for $C_{10}H_{10}O_3$; C, 67·4; H, 5·7%).

[14] T. SWAIN, *Biochem. J.* **53**, 200 (1953).
[15] J. KENDALL and J. E. BOOGE, *J. Am. Chem. Soc.* **38**, 1712 (1916).
[16] G. B. MALONE and E. E. REID, *J. Am. Chem. Soc.* **51**, 3424 (1929).
[17] A. MICHAEL, *Ber.* **34**, 3640 (1901).

Hydrolysis of (V) with 0·025N NaOH followed by acidification yielded *p*-coumaric acid (identified by paper chromatography (BEW solvent) and paper electrophoresis (acetate buffer). The ultraviolet and infrared spectra of (V) were consistent with it being methyl *p*-coumarate (lit. m.p. 137°[18]).

Reaction with caffeic acid

Chromatographic resolution (cellulose column, BEW solvent) of the mixture obtained by reacting caffeic acid (1·02 g) in methylene chloride (20 ml) with boron trichloride and methanol gave a fluorescent compound (VI, 0·62 g) which, after recrystallization from aqueous ethanol, had m.p. 152° (lit. m.p. for methyl caffeate is 152°–3[19]). (Found: C, 61·7; H, 5·0. Calc. for $C_{10}H_{10}O_4$; C, 61·9; H, 5·2%). Alkaline hydrolysis of (VI) yielded caffeic acid, which was identified by paper chromatography (BEW solvent) and paper electrophoresis (acetate buffer). The infrared and ultraviolet spectra confirmed the identification of (VI) as methyl caffeate.

Reaction with Sinapic Acid

Sinapic acid (2·13 g) in methylene chloride (40 ml) was treated with boron trichloride and methanol. The mixture was fractionated on a cellulose column (BEW solvent) and a fluorescent compound (VII, 1·01 g) obtained which was further purified on thick paper chromatograms. Hydrolysis of (VII) with N H_2SO_4 (2 hr, 100°) produced a phenolic compound which formed a brown molybdate complex and which tended to streak on paper chromatograms and electrophoretograms but nevertheless had the characteristics expected of a trihydroxycinnamic acid.

Treatment of (VII) with acetic anhydride and anhydrous zinc chloride yielded an acetate which, after repeated recrystallizations from glacial acetic acid and finally from a chloroform–light petroleum (b.p. 60–80°) mixture, had m.p. 170° (lit. m.p. for methyl 3,4,5-triacetoxycinnamate is 168°[20]). (Found: C, 57·4; H, 4·8. Calc. for $C_{16}H_{16}O_8$: C, 57·1; H, 4·8%).

Reactions with Ferulic, 3,4-Dimethoxycinnamic and Chlorogenic Acids

Approximately 0·05 g of each of the above compounds in methylene chloride (*ca.* 20 ml) was allowed to react with boron trichloride and methanol. The reaction mixtures were then placed directly on paper chromatograms (BEW and EAW solvents) and electrophoretograms (borate, molybdate and acetate buffers). In all three cases, the major product was methyl caffeate (VI).

Reaction with Coffee Bean Powder

The green beans were frozen in liquid nitrogen and then ground to a fine powder. This was dried at 60° under reduced pressure and then treated with the halide and methanol. The concentrated reaction products were taken up in chloroform and shown to contain methyl caffeate by chromatographic analysis.

[18] T. ZINCKE and F. LEISSE, *Ann. Chem. Liebigs* **322**, 220 (1902).
[19] F. MAUTHNER, *J. prakt. Chem.* **142**, 33 (1935).
[20] T. BOEHM and K. W. ROSENMUND, *Ann. Chem. Liebigs.* **437**, 125 (1924).

Reaction with Antirrhinum majus *Cinnamic Acid Derivatives*

An aqueous methanolic (80%) extract of flowers from this plant was concentrated to dryness at 60° under reduced pressure. Treatment and examination, as with the coffee bean powder, showed that methyl *p*-coumarate (V) and methyl caffeate (VI) had been formed.

Acknowledgements—We are indebted to Dr. E. C. Bate-Smith and Dr. T. Swain for their interest, to Mr. P. J. Gardner for the molecular weight determination and to the Agricultural Research Council and the United States Department of Agriculture for financial assistance.

B10

Reprinted from
"Enzyme Chemistry of Phenolic Compounds"
PERGAMON PRESS
OXFORD · LONDON · NEW YORK · PARIS
1963
Printed in Great Britain by J. W. Arrowsmith Ltd., Bristol

CHAPTER 7

HYDROLYTIC ENZYMES

J. B. PRIDHAM

Chemistry Department, Royal Holloway College, University of London,
Englefield Green, Surrey

THE HYDROLYTIC cleavage of covalent bonds such as C—O (esters and glycosides), C—N (proteins and peptides) and O—P (phosphates) is catalysed by a group of hydrolytic enzymes, or hydrolases. In many instances, these enzymes can also effect synthesis by a reversal of their normal hydrolytic action.

This review is intended to cover in general terms, the reaction of plant hydrolases with phenolic compounds and their derivatives. No enzymes in this group have so far been discovered which will react with the aromatic nuclei of phenolics. In fact, the hydrolytic cleavage of C—C bonds is rare and only occurs with some aliphatic carbon chains.[1] Hydrolases will, however, react with substituted phenolic hydroxyl groups and other substituted functional groups which may be present in phenolic compounds. Thus suitable phenolic derivatives will act as substrates for aryl sulphatases, esterases and glycosidases.

ARYL SULPHATASES

These enzymes occur mainly in animals and microorganisms and are, at present, of no great interest to workers in the plant phenolics field as naturally occurring phenolic sulphates such as persicarin[2] and persicarin-7-methyl ether[3] are quite rare. Much of the work on the sulphatases has been carried out by Dodgson and Spencer and their associates.[4] There is little reason to suppose that the biosynthesis of sulphates occurs by a reversal of aryl sulphatase activity. Activated sulphate derivatives such as adenosine-3′-phosphate-5′-sulphatophosphate (PAPS)[5] are more likely to be involved in such reactions *in vivo*.

ESTERASES

The true esterases, i.e. those which hydrolyse simple esters are common in animal tissues but are infrequently found in higher plants, although wheat germ[6] and citrus esterases[7] have been reported. Lipases, the group of esterases which hydrolyse water-insoluble esters, are widespread in plant tissues.

Aspergillus niger and other moulds yield mixtures of esterases known as *tannases*. These preparations have been particularly useful to organic chemists who are interested in the structures of hydrolysable tannins. Crude tannase from *A. niger* will hydrolyse both aliphatic and depside ester linkages but the preparation shows a high specificity towards the acyl moiety of the ester. For example, it will hydrolyse the methyl esters of 3,4,5-trihydroxy-, 3,4-dihydroxy- and 3,5-dihydroxy-benzoic acids but not those of salicylic acid and 2,4-dihydroxy- and 2,5-dihydroxy-benzoic acids.[8,9]

Toth and Barsony[10] claimed to have isolated a specific depsidase from crude tannase by chromatography. Haslam *et al.*[11] found evidence for the existence of a separate aliphatic esterase and a depsidase in tannase but were unable to achieve any separation of these activities. The Sheffield group[11] were, however, able to obtain a tannase preparation free from glycosidases by ion-exchange chromatography. This was used to investigate the structures of gallotannins[12] and it was clearly shown that they had glucose cores esterified with gallic acid residues and not oligosaccharide cores as claimed by White[13] and Grassmann *et al.*[14]

Nierenstein[15] claimed that esters could be synthesized by a reversal of tannase action. Thus he was able to form *m*-digallic acid by the condensation of two molecules of gallic acid. The physiological significance of this reaction is doubtful. Ester linkages are more likely to be synthesized *in vivo* via activated acyl derivatives. Some fungi can utilize tannin as a carbon source and here the presence of a tannase is obviously important. Tannases have so far not been reported in higher plants.

GLYCOSIDASES

The discussion with this group of enzymes will be chiefly limited to the simple glycosidases, i.e. those which hydrolyse alkyl and aryl glycosides

$$\text{Glycosyl—OR} + \text{EH} \rightleftarrows \text{Glycosyl—E} + \text{R—OH}$$

$$\text{Glycosyl—E} + \text{HO—R}' \rightleftarrows \text{Glycosyl—OR}' + \text{EH}$$

FIG. 1.

and low molecular weight oligosaccharides. The enzymes which hydrolyse polysaccharides and those which are involved in transfer reactions with nucleotide derivatives of sugars, such as uridine diphosphate glucose (UDPG) may also be regarded as glycosidases. These, however, will not be dealt with in any detail.

The enzymic hydrolysis of glycosides occurs by fission of the bond between C—1 of the glycone and the glycosidic oxygen atom.[16] The overall reaction is shown in Fig. 1. R in the glycosyl donor molecule may

be an alkyl, aryl or glycosyl group. If R′ in the acceptor molecule is H then normal hydrolytic cleavage of the glycoside occurs with the liberation of free reducing sugar. Where R′ is an alkyl or glycosyl group a new glycoside bond is formed by transglycosylation. Most glycosidases exhibit competing hydrolytic and transferase activities, hydrolysis really being a special form of transglycosylation with water acting as the acceptor molecule.

Hydrolase Activity

The existence of glycoside-hydrolysing enzymes has been recognized for 150 years although a great many questions remain unanswered regarding their mode of action. This is mainly because they frequently occur in complex mixtures which are difficult to fractionate. At the present time only one crystalline glycosidase is known, namely the β-galactosidase from *E. coli.*[17]

The most common sources of glycosidases are the *Rosaceous* plants and in particular sweet almond seeds from which almond emulsin is obtained. The properties of this complex mixture of enzymic proteins has been extensively reviewed.[18–22] The main enzymes present in the emulsin are β-D-glucopyranosidase, β-D-galactopyranosidase, α-D-galactopyranosidase, and α-D-mannopyranosidase. There is some controversy regarding the β-glucosidase and β-galactosidase. Helferich [23] believed that a single enzyme was responsible for both activities. This was disputed[19] but recent evidence[24] again suggests that one active site is responsible for both activities. Almond emulsin also contains minor β-D-glucopyranuronosidase and 2-acetamido-2-deoxy-β-D-glucopyranosidase components, and will hydrolyse α- and β-L-arabinopyranosides, β-D-xylopyranosides and α-D-lyxopyranosides. It is not necessary to postulate the existence of separate enzymes in the case of the pentosides as these may well be hydrolysed by the major hexosidase components with the corresponding structural specificities.

In general terms the specificity of a glycosidase is directed towards four structures in the substrate:

1. Configuration at the anomeric carbon atom.
2. Shape of the sugar ring.
3. Arrangement and substitution of the hydroxyl groups on the sugar ring.
4. Structure of the aglycone.

Aglycone specificity is normally much less stringent than glycone specificity.

In the case of purified almond β-glucopyranosidase the enzyme will not hydrolyse α-glucopyranosides, β-glucofuranosides or β-thioglucopyranosides. Absolute specificity for the configuration of the groups on C—2, C—3, C—4 and C—5 exists and the enzyme will not tolerate substitution of the hydroxyl groups on C—2, C—3 or C—4. Substitutions at C—5 or C—6 affect the rate of hydrolysis but do not normally result in complete inhibition. In general, the larger the substituent at C—6 the slower the rate of hydrolysis. β-Glucopyranosidase has a low degree of specificity with regard to the aglycone although differences in structure do affect the rate of reaction. For example, aryl-β-glucopyranosides are usually hydrolysed more rapidly than alkyl-β-glucopyranosides and substitution of the aglycone moiety in an aryl-β-D-glucopyranoside with an electron-attracting group facilitates enzymic hydrolysis.[25] With other glucopyranosidases there are wide variations in aglycone specificities. Thus the α-D-glucopyranosidase from *Candida tropicalis*[26] will hydrolyse starch, maltose, sucrose, methyl-α-D-glucopyranoside and phenyl-α-D-glucopyranoside. *Stachybotyris atra* β-D-glucopyranosidase[27] on the other hand exhibits a high affinity for aryl-β-D-glucopyranosides.

Transferase Activity

Transglycosylation with the formation of new glycosidic bonds occurs when the acceptor molecule is an alcohol. This was first observed by

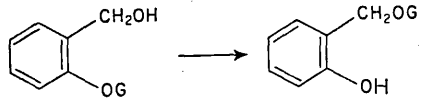


FIG. 2.

Rabaté[28] who showed that an emulsin from *Salix purpurea* would transfer glucose from the phenolic hydroxyl group of saligenin to the primary hydroxyl group (Fig. 2), and also that glucose could be transferred from phenyl-β-D-glucopyranoside to primary alcohols. Since these original observations transglycosylation reactions involving many glycosidases have been studied in great detail. This literature has recently been reviewed by Jermyn[29] and Dedonder.[30] It is interesting to note that there is always retention of configuration at C—1 of the transferred glycone moiety in these reactions.*

Glycosidases show some specificity towards acceptor molecules. Courtois and Leclerc,[32] for example, have shown that almond β-glucopyranosidase will transfer glucose from phenyl-β-D-glucopyranoside to

* (In the case of transfer from UDPG inversion at C—1 of the glucosyl residue may occur).[31]

various sugars, simple primary and secondary alcohols but not tertiary alcohols.

Miwa and other Japanese workers using a number of different glycosidases, have shown that each enzyme exibits characteristic affinities towards different acceptor molecules. This work has been summarized by Jermyn.[27]

Attempts to glucosylate phenolic hydroxyl groups using higher plant glycosidases and "low energy" potential glucose donors have failed.[35–36] In other words, phenols would appear to be rather poor acceptor molecules. Pridham,[37] however, has been able to transfer glucose from maltose to resorcinol with the formation of *m*-hydroxyphenyl-α-D-glucopyranoside and *m*-hydroxyphenyl-α-isomaltoside using an *A. niger* enzyme preparation. Other di- and tri-hydric phenols also acted as acceptors in this system. It is at present uncertain whether the enzyme responsible for this reaction falls into the class of simple glycosidases as defined on page 74. At least no dialysable co-factor appeared to be involved in the reaction and transfer from UDPG to resorcinol could not be demonstrated.

Mechanism of Glycosidase Action

The glycosidases are related in a number of ways. They are usually acidic proteins and have no prosthetic groups or coenzymes. Maximum hydrolytic and transferase activities normally occur in neutral or weakly acidic solutions and hydrolysis proceeds by hexose—0 bond fission.[16] With regard to the active sites on these enzymes, few have been examined in detail, but a variety of acidic and basic groups have been implicated At the present time there is widespread belief that the majority of simple glycosidases have a similar mechanism of action and probably function as acid-base catalysts. In other words, the glycoside undergoes attack by nucleophilic and electrophilic groups on the enzyme surface which results in electron displacement and rupture of the glycosidic bond.

A recent, attractive mechanism for *E. coli* β-galactopyranosidase activity, based on Koshland's double displacement theory[38] has been proposed by Wallenfels and Malhotra.[39] This enzyme is a typical glycosidase and pH-activity and inhibitor studies indicated that sulphydryl and imidazole groups were present on the active site. It is suggested that the enzyme-substrate complex is formed as shown in Fig. 3, the galactosyl residue being held onto the enzyme surface by hydrogen bonds between carbonyl groups of alternate peptide bonds and hydroxyl groups on the sugar. Six to seven amino acid residues are probably present between the cysteine and the histidine. Hydrolysis of the galactoside is effected by nucleophilic attack by the imidazole nitrogen on C—1 of the sugar and simultaneous hydrogen bond formation between the glycosidic oxygen atom and the sulphydryl group (Fig. 4). Bond fission occurs and free galactose or a new galactoside

is then produced by a second nucleophilic attack by the acceptor molecule (water or an alcohol). It should be noted that with this mechanism the aglycone group and the acceptor molecule occupy the same position on the enzyme surface. The two S_{N2} reactions produce a double inversion at C—1 and the β-configuration of the original galactoside is thus retained.

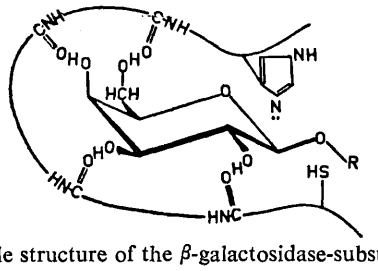


FIG. 3. A possible structure of the β-galactosidase-substrate complex.[39]

With reference to this mechanism and assuming that other glycosidases function in a similar manner, it is interesting to speculate why phenols cannot generally act as acceptor molecules in glycosylation reactions. The answer may of course be that the phenol and the glycosyl-enzyme complex are sterically incompatible. If, however, aglycones and acceptors occupy

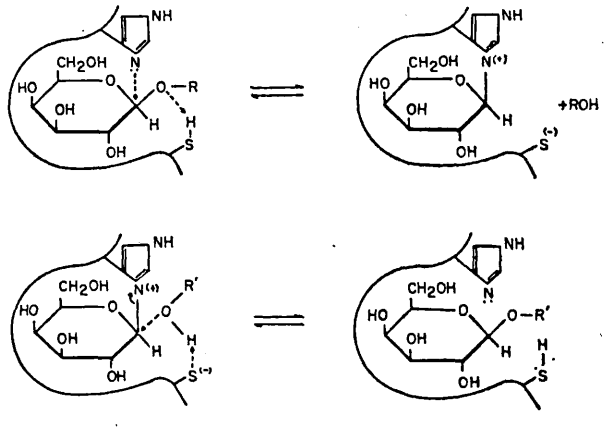


FIG. 4. Proposed mechanism for β-galactosidase action.[39]

the same position on the enzyme surface this argument may not be valid. A second possibility is based on the fact that the electron density at the oxygen atom of a phenolic hydroxyl group is lower than that of an oxygen in an alcoholic hydroxyl group. In other words, if the phenol is a weak nucleophile the formation of the new glycoside bond may be hindered.

PHYSIOLOGICAL IMPORTANCE OF GLYCOSIDASES

It is normally assumed that plant glycosidases can and do hydrolyse glycosides *in vivo*. For example, Freudenberg *et al.*[40] believe that in lignin biosynthesis the first stage is the liberation of coniferyl alcohol from coniferin by a β-glucopyranosidase. Glycosidically bound sugars may also function as energy reserves.[41] In this case one might again assume that glycosidases liberate these sugars before they are metabolized by the plant. The evidence which supports glycosidase activity *in vivo* is, however, only circumstantial and it is not known with any certainty if glycosidases and glycosides ever come into contact in normal healthy tissues. Where cells are mechanically damaged or diseased, contact is certain and subsequent hydrolysis with the liberation of antimicrobic aglycones could in these instances be an important function of glycosidases. The exact significance of other carbohydrases, such as the amylases and invertase, in plant tissues is also not clear.

The participation of simple glycosidases in the biosynthesis of glycosidic bonds in living plant tissues seems very unlikely. The equilibrium constants for reactions of the type shown in Fig. 1, normally favour hydrolysis and not synthesis. In the presence of high substrate concentration, however, a simple transfer reaction could result in glycoside synthesis. Thus 1^F-β-fructosylsucrose might well arise by the action of sucrase on sucrose in the cambial tissues of aspen.[34] In the case of the phenolic glycosides it is almost certain that biosynthesis in higher plants is effected via "high energy"[35,42] sugar derivatives such as UDPG. It is possible *in vitro* to synthesize a diglucoside from a monoglucoside using a simple glycosidase (e.g. to convert arbutin to *p*-hydroxyphenyl-β-gentiobioside with almond emulsin[36]) but, here again, *in vivo* the second glucose residue is probably derived from UDPG.[34,43]

REFERENCES

1. E. Racker, in *The Enzymes*, 2nd ed., Vol. 5, p. 313, eds. P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York (1961).
2. H. Tatsuta and Y. Ochii, *J. Chem. Soc. Japan* **75**, 241 (1954).
3. H. Tatsuta, *J. Chem. Soc. Japan* **75**, 939 (1954).
4. See references in *Biochemists' Handbook*, p. 270, ed. C. Long, Spon, London (1961).
5. J. Baddiley and J. G. Buchanan, *Quart. Revs.* **12**, 171 (1958).
6. T. P. Singer and B. H. J. Hofstee, *Arch. Biochem. Biophys.* **18**, 229 (1948).
7. E. F. Jansen, R. Jang and L. R. MacDonnell, *Arch. Biochem. Biophys.* **15**, 415 (1947).
   E. F. Nansen, M-D. F. Nutting and A. K. Balls, *J. Biol. Chem.* **175**, 975 (1948).
8. H. Dyckerhoff and R. Armbruster, *Z. Physiol. Chem.* **219**, 38 (1933).
9. E. Haslam, R. D. Haworth, K. Jones and H. J. Rogers, *J. Chem. Soc.* 1829 (1961).
10. G. Toth and G. Barsony, *Enzymologia* **11**, 19 (1943–45).
11. E. Haslam, R. D. Haworth, S. D. Mills, H. J. Rogers, R. Armitage and T. Searle, *J. Chem. Soc.* 1836 (1961).

12. R. ARMITAGE, G. S. BAYLISS, J. W. GRAMSHAW, E. HASLAM, R. D. HAWORTH, K. JONES, H. J. ROGERS and T. SEARLE, *J. Chem. Soc.* 1842 (1961).
13. T. WHITE, *The Chemistry of Vegetable Tannins*, p. 13, Soc. Leather Trades' Chemists, Croydon (1956).
14. W. GRASSMANN, G. STIEFENHOFER and H. ENDRES, *Ber.* **89**, 454 (1956).
15. M. NIERENSTEIN, *Biochem. J.* **26**, 1093 (1932).
16. C. A. BUNTON, T. A. LEWIS, D. R. LLEWELLYN, H. TRISTRAM and C. A. VERNON, *Nature, Lond.* **174**, 560 (1954).
D. E. KOSHLAND and S. S. STEIN, *J. Biol. Chem.* **208**, 138 (1954).
S. E. SPRINGHORN and D. E. KOSHLAND, 128th Meeting ACS, abstracts, p. 37C (1955).
17. K. WALLENFELS and M. L. ZARNITZ, *Angew Chem.* **69**, 482 (1957).
K. WALLENFELS, M. L. ZARNITZ, G. LAULE, H. BENDER and M. KESER, *Biochem. Z.* **331**, 459 (1959).
A. S. L. HU, R. G. WOLFE and R. J. REITHEL, *Arch. Biochem. Biophys.* **81**, 500 (1959).
18. W. W. PIGMAN, *Adv. Enzymol.* **4**, 41 (1944).
19. A. GOTTSCHALK, *Adv. Carbohydrate Chem.* **5**, 60 (1950).
20. S. VEIBEL, in *The Enzymes*, 1st ed., Vol. 1, Pt. I, pp. 583, 621, eds. J. B. Sumner and K. Myrbäck, Academic Press, New York (1950).
21. H. BAUMANN and W. PIGMAN in *The Carbohydrates*, p. 562, ed. W. Pigman, Academic Press, New York (1957).
22. A. Gottschalk in *Encylopedia of Plant Physiology*, Vol. 6, p. 107, ed. W. Ruhland, Springer-Verlag, Berlin (1958).
23. B. HELFERICH, *Ergeb. Enzymforsch.* **7**, 83 (1938).
24. R. HEYWORTH and P. G. WALKER, *Biochem. J.* **83**, 331 (1962).
25. R. L. NATH and H. RYDON, *Biochem. J.* **57**, 1 (1954).
26. T. SAWAI, *J. Biochem., Tokyo* **44**, 49 (1958).
27. M. A. JERMYN, *Aust. J. Biol. Sci.* **8**, 577 (1955).
28. M. RABATÉ, *Bull. Soc. Chim. Biol.* **17**, 572 (1935).
29. M. A. JERMYN, *Rev. Pure Applied Chem.* **11**, 92 (1961).
30. R. A. DEDONDER, *Ann. Rev. Biochem.* **30**, 347 (1961).
31. W. Z. HASSID, E. F. NEUFELD and D. S. FEINGOLD, *Proc. Natl. Acad. Sci. U.S.* **45**, 905 (1959).
32. J. E. COURTOIS and M. LECLERC, *Bull. Soc. Chim. Biol.* **38**, 365, (1956).
33. C. E. CARDINI and T. YAMAHA, *Nature, Lond.* **182**, 1446 (1958).
34. J. B. PRIDHAM, *Biochem. J.* **76**, 13 (1960).
35. J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **74**, 42P (1960).
36. J. D. ANDERSON, L. HOUGH and J. B. PRIDHAM, *Biochem. J.* **77**, 564 (1960).
37. J. B. PRIDHAM, *Chem. and Ind.* 1172 (1961).
38. D. E. KOSHLAND in *The Enzymes*, 2nd ed., Vol. 1, p. 305, eds. P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York (1959).
39. K. WALLENFELS and O. P. MALHOTRA in *The Enzymes*, 2nd ed., Vol. 4, p. 409, eds. P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York (1960).
40. K. FREUDENBERG, H. REZNIK, H. ROSENBERG and D. RASENACK, *Ber.* **85**, 641 (1952)
41. W. PFEFFER, *Pflanzen. Physiol.* **1**, 492 (1897).
M. BRIDEL, *Rev. Gén. Sci. Pure Appl.* **37**, 134 (1926).
T. WEEVERS, *Jb. Wiss. Bot.* **39**, 229 (1904).
42. T. YAMAHA and C. E. CARDINI, *Arch. Biochem. Biophys.* **86**, 127 (1960).
43. T. YAMAHA and C. E. CARDINI, *Arch. Biochem. Biophys.* **86**, 133 (1960).

Biochem. J. (1963) **87**, 218
Printed in Great Britain

The Biosynthesis of Phenolic Glucosides in Plants

BY J. B. PRIDHAM AND MARGARET J. SALTMARSH
Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 12 *October* 1962)

The first claim that phenolic glucosides were formed when phenols were fed to plants was made by Ciamician & Ravenna (1916). These workers reported that saligenin (*o*-hydroxybenzyl alcohol) was converted into salicin (*o*-hydroxymethylphenyl β-D-glucopyranoside) by maize seedlings. Other feeding experiments with a variety of phenols have since been carried out and it is now clear that glucosylation of phenols is a common reaction in plant tissues. These studies, leading to the implication of uridine diphosphate glucose in the reaction, have been reviewed by Pridham (1960*a*). More recent advances have included feeding coumarin and *o*-coumaric acid to white clover with the formation of melilotyl- and *o*-coumaryl-glucosides respectively (Kosuge & Conn, 1959) and allowing the leaves of various plant species to take up cinnamic acid derivatives (Harborne & Corner, 1961). In this latter case the corresponding glucose esters were the main products, but with caffeic acid the 3- and 4-β-glucosides were also formed. Yamaha & Cardini (1960*a*) have now studied in some detail the enzyme from wheat germ which is specific for the formation of glucosides from uridine diphosphate glucose and phenols with 1,4-dihydroxy groupings (cf. Cardini & Leloir, 1957; Pridham & Saltmarsh, 1960) and the enzyme which transfers glucose from uridine diphosphate glucose to arbutin with the formation of a β-gentiobioside (Yamaha & Cardini, 1960*b*; cf. Pridham, 1957, 1960*b*; Anderson, Hough & Pridham, 1960). The wheat-germ enzyme will also transfer glucose from adenosine diphosphate glucose to quinol (Trivelloni, Recondo & Cardini, 1962). Marsh (1960) has transferred glucuronic acid from uridine diphosphate glucuronic acid to quercetin using an enzyme from French beans and Barber (1962) has converted this flavonol into the glycoside rutin with an enzyme preparation from mung beans in the presence of uridine diphosphate glucose (or thymidine diphosphate glucose) and thymidine diphosphate rhamnose. The formation of phenolic α-glucosides in the absence of nucleotides has been observed with *Aspergillus niger* extracts, maltose being used as a glucose donor (Pridham, 1961). Acidic derivatives of phenolic glucosides are also produced when broad-bean seeds are treated with simple phenols (Pridham & Saltmarsh, 1962).

This study has been undertaken to establish the nature of the glucosides formed by feeding simple phenols to the broad bean (*Vicia faba*) and to compare these products with those obtained from experiments *in vitro*. All possible steps have been taken to determine accurately the structures of the phenolic glucosides produced, but in some cases low yields or instability of the compounds, or both, have prevented a complete chemical and physical identification. We consider that inadequate characterization may have led to error in some previous publications. For example, some doubt exists about the product of glucosylation of saligenin, where isomeric monoglucosides could be formed by reaction with the phenolic or the primary hydroxyl group.

A preliminary account of this work has been published (Pridham & Saltmarsh, 1960).

METHODS

General methods. M.p. values are uncorrected. Ultraviolet-absorption spectra were measured with a Unicam SP.500 spectrophotometer (1 cm. cell). Free phenolic hydroxyl groups were detected by a bathochromic shift in the presence of base (Mansfield, Swain & Nordstrom, 1953) and ene-diol groupings were revealed by a hypsochromic shift on subsequent addition of borate ions to the alkaline

solution (Swain, 1954). Infrared measurements were made with a Unicam SP. 100 double-beam spectrophotometer. In the enzyme-catalysed reactions control incubations with boiled-enzyme preparations were always carried out.

Paper chromatography. Phenolic compounds and sugars were examined on Whatman no. 1 and no. 3 papers respectively by the descending technique. The solvent systems were: *A*, butan-1-ol–ethanol–water (40:11:19, by vol.); *B*, ethyl acetate–acetic acid–water (9:2:2, by vol.); *C*, *NN*-dimethylformamide–benzene (4:1, v/v; stationary phase), light petroleum (b.p. 60–80°; mobile phase) (Wickberg, 1958); *D*, *NN*-dimethyl sulphoxide–benzene (4:1, v/v; stationary phase), di-isopropyl ether (mobile phase) (Wickberg, 1958). Molybdate-buffered papers were prepared as described by Pridham (1959). The stability of glucosides to oxidation was investigated by spotting these compounds on the starting lines of chromatograms, spraying with an aqueous M-$FeCl_3$ solution and allowing them to dry at room temperature. These chromatograms were then developed with solvent *A* and treated with spray *A*.

Paper electrophoresis (*see Pridham*, 1959). Whatman no. 3 paper was used with the following buffer solutions: *A*, 0·2M-sodium borate, pH 10·0; *B*, 0·05M-glycine, pH 10·0; *C*, 8 mM-ammonium molybdate, pH 5·2; *D*, 0·2M-sodium acetate, pH 5·2.

Location of compounds. Phenolic compounds were detected on paper chromatograms and electrophoretograms by the use of diazotized *p*-nitroaniline–NaOH (spray *A*; Swain, 1953) and by examination under u.v. light, a Chromatolite (Hanovia Ltd., Slough, Bucks.) being used. *p*-Anisidine hydrochloride (spray *B*; Hough, Jones & Wadman, 1950) was used for reducing sugars and urea hydrochloride (spray *C*; Isherwood, 1954) for ketoses.

Feeding experiments. Vicia faba (var. Johnson's Longpod) seeds were steeped in water (24 hr.) and then placed between layers of cotton wool soaked in an aqueous solution (1%, w/v) of the phenol for 3 days. Treatment of 6-day-old maize seedlings (*Zea mays*, var. Golden Harvest) with saligenin was carried out by a similar procedure. With willow (*Salix daphnoides*), cut ends of shoots were placed in aqueous solutions of saligenin (1%, w/v) for 48 hr. before examination.

Isolation of phenolic glucosides. The testas were removed from the treated bean seeds and the cotyledons and embryos macerated with aqueous (90%, v/v) methanol. The resulting slurries were centrifuged (3000 rev./min. for 15 min. at 5°) and the supernatants were then concentrated and examined on paper chromatograms (solvents *A* and *B*) for the presence of phenolic glucosides (u.v. light and spray *A*). Maize seedlings and willow shoots were extracted by a similar method. Fractionations of the extracts were achieved on cellulose columns or on Whatman no. 3 paper (solvent *A*).

Acidic hydrolysis. The glucosides were heated with 1·5 N-HCl at 100° for 4 hr. and the solutions then evaporated to dryness over NaOH pellets in a vacuum desiccator. The hydrolysates were examined on paper chromatograms with solvents *A* and *B* (sprays *A* and *B*).

Enzymic hydrolysis. The glucosides were treated with almond β-glucosidase (Sigma Chemical Co.; 1%, w/v, in 0·02M-sodium acetate buffer, pH 5·5) at 27° for 48 hr. and the digests then examined chromatographically in the same way as the acidic hydrolyses. Complete hydrolysis of compound (XV) was effected with yeast invertase (British Drug Houses Ltd. concentrate, 0·2 ml.) in 0·1M-sodium phosphate buffer, pH 7·5 (0·2 ml.), at 25° for 30 min.

Methylation of compound (VI). Diazomethane (10 ml.) prepared by Nierenstein's (1930) method was added to freeze-dried (VI) (10 mg.) in dry methanol (10 ml.) and kept at 0° for 18 hr. Acetic acid was then added dropwise until the yellow colour of the solution disappeared. After concentration to dryness the methylated product was heated with methanolic (7%, w/v) HCl and evaporated to dryness again over NaOH pellets in a vacuum desiccator. The hydrolysate was examined on paper chromatograms with solvents *C* and *D* and compared with authentic 2,4- and 2,5-dimethoxyphenols.

Preparation of 2,5-dimethoxyphenol sulphonate. Conc. H_2SO_4 (3 ml.) was added to quinol dimethyl ether (6 g.) and the mixture heated on an oil bath at 110–120° for 1 hr. The reaction mixture, after cooling, was poured into ice–water and $NaHCO_3$ added to give pH 6–7. The solution was then heated to boiling point and saturated with NaCl and filtered. On cooling, white crystals of sodium 2,5-dimethoxybenzenesulphonate were obtained The *p*-toluidine derivative had m.p. 203° [Gallent (1958) found m.p. 202–203°].

Preparation of 2,5-dimethoxyphenol. Sodium 2,5-dimethoxybenzenesulphonate (6 g.) was fused with NaOH (10 g.) and KOH (4 g.) in an iron crucible. The fusion product was dissolved in water, the pH adjusted to 5–6 with conc. H_2SO_4 and the solution extracted with ether. Concentration of the ethereal extract gave an oil in low yield. This compound behaved as a typical dimethoxyphenol in solvents *A*, *C* and *D* and gave a blue azo dye with spray *A*.

Separation of compounds (IX) *and* (X). Initial purification of the mixed glucosides was achieved in thick-paper chromatograms with solvent *A*. The resulting mixed fraction (IX and X) was then resolved by paper electrophoresis with buffer *A*, compound (IX) having M_{SA} (rate of movement relative to salicylic acid; Pridham, 1959) 0·29 and (X) M_{SA} 0. The bands were eluted from the electrophoretograms with water.

o-*Hydroxybenzyl β-glucoside.* This was prepared by the method of Anderson *et al.* (1960).

p-*Hydroxy-and* m-*hydroxy-benzyl β-glucosides.* These were synthesized from glucose and the corresponding phenol by the biochemical method of Bourquelot & Hérissey (1913).

Wheat-germ enzyme. This was prepared by Yamaha & Cardini's (1960*a*) method and the fraction I obtained by these workers used for the synthesis of glucosides.

Broad-bean enzyme. Dormant bean seeds with testas removed (35 g.) were macerated with 0·05M-sodium phosphate buffer, pH 7·0 (105 ml.), at 0°. The slurry was left to stand for 2 hr. at 5°, centrifuged (16 000 *g* at 0° for 20 min.) and the supernatant was dialysed against 0·05M-sodium phosphate buffer, pH 7·0, at 5°.

Glucose transfer to phenols in vitro. The phenol (10 mg.) was incubated with UDP-glucose (6 mg.), enzyme (wheat germ or broad bean; 0·1 ml.) and 0·05M-tris–HCl buffer (0·1 ml., pH 7·4) at 37° under toluene. The final pH of the reaction mixture was 7·2. Paper-chromatographic examination (solvents *A* and *B*) was carried out after incubation for 5 hr.

Transfer of glucose to phenols was also achieved by incubating (at 37°) wheat-germ enzyme (0·1 ml.) with 0·05M-tris–HCl buffer (0·1 ml., pH 7·4), ATP (2·4 mg.), UTP (1·4 mg.), α-D-glucose 1-phosphate (2·1 mg.) and the phenol (1·3 mg.) in the presence of $MgCl_2$ (0·5 mg.) and NaF

(2·0 mg.). The reaction mixtures were again examined on paper chromatograms (solvents *A* and *B*) after 5 hr. In one experiment α-D-glucose 1-phosphate in the above reaction mixture was replaced by α-D-galactose 1-phosphate in an attempt to transfer galactose to quinol and resorcinol.

Resorcinol (10 mg.) was also incubated (at 37°) separately with α-D-glucose 1-phosphate, methyl α-D-glucoside, maltose and cellobiose (6 mg. each) in the presence of wheat-germ enzyme (0·1 ml.) and 0·05 M-tris–HCl buffer (0·1 ml., pH 7·4) and the digests were examined chromatographically (solvents *A* and *B*) after 5 hr.

Attempted transfer of glucose to phenols with almond β-glucosidase. β-Glucosidase (Sigma Chemical Co.; 0·2 ml.; 1%, w/v, in 0·05 M-sodium acetate buffer, pH 5·6) was incubated separately with arbutin (14 mg.), cellobiose (16 mg.), methyl β-D-glucoside (10 mg.) and D-glucose (9 mg.) in the presence of quinol, resorcinol or 1,2,4-trihydroxybenzene (1·4 mg. each) at 27°. The digests were examined on paper chromatograms after 8, 24 and 72 hr. (solvents *A* and *B*).

Attempted transfer of fructose to phenols with yeast invertase. The phenol (10 mg.), sucrose (20 mg.), invertase (British Drug Houses Ltd. concentrate; 0·2 ml.) and 0·1 M-sodium phosphate buffer, pH 7·5 (2 ml.), were incubated at 25° and samples taken and examined chromatographically (solvents *A* and *B*) after 5 min., 3 hr. and 20 hr.; a high pH was chosen in the hope that increased ionization of the phenolic hydroxyl groups would facilitate the transfer reaction. Only with saligenin was a glycosylated product (XV) obtained and this was isolated from the reaction mixture by chromatography on thick paper (solvent *A*). The hydrolysis products obtained on heating (XV) with 0·01 N-H_2SO_4 at 100° for 10 min. were examined on paper chromatograms (solvents *A* and *B*).

RESULTS

Feeding experiments

Preliminary experiments showed that broad-bean seeds that had been allowed to germinate for 24 hr. before feeding with phenols gave the highest yields of glucosides, in comparison with organs of the mature plant. In addition, seed extracts contained fewer interfering phenolic substances than extracts of the green parts of the plant and this facilitated the isolation of the glucosylated products. The main properties of the glucosides formed in the feeding experiments are summarized in Table 1. Quinol gave a higher yield of the corresponding monoglucoside, arbutin (I; 300 mg. from 110 g. dry wt. of seeds) than any other phenol that was used for feeding. Arbutin, unlike the other glucosides, was obtained in a crystalline form from water: m.p. and mixed m.p. 199°, $[\alpha]_D^{17} - 62°$ (*c* 1·8 in water) (Found: C, 49·7; H, 6·3. Calc. for $C_{12}H_{16}O_7,H_2O$: C, 49·7; H, 6·2%), and was also characterized by paper chromatography and electrophoresis against authentic arbutin and by comparative u.v. and i.r. spectrophotometry. The penta-acetate of (I) had m.p. and mixed m.p. 146·5° (Found: C, 54·3; H, 5·6. Calc. for $C_{22}H_{26}O_{12}$: C, 54·8; H, 5·4%). The i.r. spectrum of the penta-acetate was identical with that of authentic *p*-hydroxyphenyl β-D-glucopyranoside penta-acetate. The unequivocal identification of (I) supports the structures proposed for the other glucosides, which could only be examined by less rigorous methods.

The mono β-glucoside (II), obtained by feeding with resorcinol, was isolated as a freeze-dried powder and had paper-chromatographic and electrophoresis properties identical with those of authentic *m*-hydroxyphenyl β-glucoside.

Similarly, compound (III), which was derived from catechol, was electrophoretically and chromatographically indistinguishable from *o*-hydroxyphenyl β-glucoside.

Two mono β-glucosides, compounds (IV) and (V), in the approximate proportions of 3:1 (as judged visually from paper chromatograms) were produced in low yields by seeds fed with pyrogallol. Compound (IV) gave a red–brown colour on molybdate-treated paper chromatograms and on molybdate electrophoretograms (buffer *C*). This reaction, in conjunction with the R_F and M_{SA} values (Table 1) is highly characteristic of compounds containing an ene-diol grouping (Pridham, 1959). The spectrum of (IV) is also characteristic of an ene-diol and the structure of this compound must therefore be 2,3-dihydroxyphenyl β-glucoside. Compound (V) gave negative ene-diol reactions and is presumably 2,6-dihydroxyphenyl β-glucoside.

Extracts prepared after feeding 1,2,4-trihydroxybenzene to bean seeds contained three compounds, (VI), (VII) and (VIII), in the approximate proportions of 6:2:1; which behaved as monoglucosides on paper chromatograms and electrophoretograms. Insufficient amounts of (VIII) could be obtained for hydrolysis studies and this compound seemed to be much more unstable than (VI) or (VII). Compound (VII) behaved as an ene-diol in the presence of molybdate ions, unlike compound (VI). Attempts to detect the ene-diol grouping in (VII) by spectrophotometry failed owing to the instability of the compound. Treatment of all three glucosides with dilute aqueous ferric chloride solution showed that only (VI) was not oxidized, and therefore presumably did not possess a potential quinonoid structure. Methylation followed by hydrolysis of (VI) yielded a compound that co-chromatographed with 2,4-dimethoxyphenol and gave an azo dye of the same colour with spray *A*. This methylated phenol could be easily distinguished from 2,5-dimethoxyphenol by the use of 'two organic-phase' chromatography (solvents *C* and *D*). The evidence strongly suggests that compounds (VI) and (VII) are 2,4- and 3,4-dihydroxyphenyl β-glucosides respectively. Compound (VIII) may well be the 2,5 isomer.

Table 1. *Properties of glucosides isolated from bean seeds fed with various phenols*

A, *B*, *C* and *D* under M_{SA} refer to buffers described in the Methods section.

Compound	R_F Solvent *A*	R_F Solvent *B*	M_{SA} *A*	M_{SA} *B*	M_{SA} *C*	M_{SA} *D*	$\lambda_{max.}$ Water	$\lambda_{max.}$ NaOH	$\lambda_{max.}$ Borate	Acidic and β-glucosidase hydrolysis products	Colour with diazotized *p*-nitroaniline–NaOH
(I) Arbutin; *p*-hydroxyphenyl β-glucoside	0·47	0·45	0·22	0·16	0	0	278	—	—	Glucose, quinol	Purple
(II) *m*-Hydroxyphenyl β-glucoside	0·50	0·47	0·43	0·34	0	0	271	—	—	Glucose, resorcinol	Red
(III) *o*-Hydroxyphenyl β-glucoside	0·61	0·59	0·40	0·41	0	0	—	—	—	Glucose, catechol	Red
(IV) 2,3-Dihydroxyphenyl β-glucoside	0·37 (0·03)*	0·37 (0·12)*	0·56	0·43	0·69	0·08	270	283	277	Glucose, pyrogallol	Prussian blue
(V) 2,6-Dihydroxyphenyl β-glucoside	0·52 (0·55)*	0·61 (0·62)*	0·46	0·33	0	0	276	281	—	Glucose, pyrogallol	Green
(VI) 2,4-Dihydroxyphenyl β-glucoside	0·51 (0·56)*	0·40 (0·45)*	0·46	0·42	0	0	284	292	—	Glucose, 1,2,4-trihydroxybenzene	Blue–green
(VII) 3,4-Dihydroxyphenyl β-glucoside	0·40 (0·06)*	0·30 (0·05)*	0·50	—	0·77	0·05	280	291	—	Glucose, 1,2,4-trihydroxybenzene	Pale blue
(VIII) 2,5-Dihydroxyphenyl β-glucoside (?)	0·33 (0·34)*	0·49 (0·50)*	0·78	—	0·45	0·44	271	—	—	—	Pale blue
(IX) *o*-Hydroxybenzyl β-glucoside	0·59	0·50	0·29	0·27	0	0	274	297	—	Glucose, saligenin	Red
(X) Salicin; *o*-hydroxymethyl-phenyl β-glucoside	0·55	0·48	0	0	0	0	268	268	—	Glucose, saligenin	No reaction
(XI) *m*-Hydroxybenzyl β-glucoside	0·58	0·49	0·14	—	0	0	276	295	—	Glucose, *m*-hydroxybenzyl alcohol	Red
(XII) *p*-Hydroxymethyl-phenyl β-glucoside	0·49	0·52	0	—	—	0	264	264	—	Glucose, *p*-hydroxybenzyl alcohol	No reaction
(XIII) *p*-Hydroxybenzyl β-glucoside	0·43	0·47	0·44	—	—	0	—	—	—	—	Red–purple
(XIV) *p*-Nitropheny β-glucoside	0·60	0·71	0	—	—	0	294	294	—	Glucose, *p*-nitrophenol	No reaction

* R_F on molybdate-treated papers.

o-Hydroxybenzyl β-glucoside (IX) was the main product formed on feeding saligenin to germinating beans. Compound (IX) and authentic *o*-hydroxybenzyl β-glucoside (Anderson *et al.* 1960) behaved identically on paper chromatograms and electrophoretograms and gave the same red coloration with the diazonium spray reagent (*A*). A trace of salicin (X) was also detected as a u.v.-absorbing spot on a paper electrophoretogram (buffer *A*) of the seed extract. Failure of compound (X) to react with spray *A* indicated the absence of a free phenolic hydroxyl group, as did u.v. spectrophotometry, which on the other hand showed the presence of a free phenolic hydroxyl group in the major product (IX) (cf. Rabaté & Ramart-Lucas, 1935). The electrophoresis and chromatographic behaviour of compound (X) was identical with that of authentic salicin. *o*-Hydroxybenzyl β-glucoside was also the major glucoside formed when willow shoots and maize seedlings were allowed to take up saligenin. In the latter case no salicin was detected; with the willow, salicin was present but was, of course, also found in the control shoots.

m-Hydroxybenzyl alcohol was likewise converted into the corresponding alcoholic glucoside (*m*-hydroxybenzyl β-glucoside, XI) by the bean. No definite evidence was obtained for the presence of the isomeric phenolic glucoside in the seed extracts. The glucoside obtained by incubating *m*-hydroxybenzyl alcohol with excess of D-glucose and β-glucosidase (Bourquelot & Hérissey, 1913) gave the same colour with spray reagent *A* and had identical chromatographic, electrophoresis and spectral properties with compound (XI). Hydrolysis of both compounds gave glucose and *m*-hydroxybenzyl alcohol.

In contrast, *p*-hydroxybenzyl alcohol was converted largely into *p*-hydroxymethylphenyl β-glucoside (XII). The absence of a free phenolic hydroxyl group in this compound was evident from electrophoresis and spectrophotometric studies. A small amount of a compound (XIII) that gave a pink–purple colour with spray *A* also accompanied (XII). Insufficient quantities of (XIII) were present for detailed studies but it behaved in an identical manner with the glucoside obtained from *p*-hydroxybenzyl alcohol (by Bourquelot & Hérissey's, 1913, method) on paper chromatograms and electrophoretograms. The synthetic compound had $\lambda_{max.}$ 279 mμ shifting to 290 mμ on addition of alkali, and on hydrolysis with acid and β-glucosidase yielded glucose and *p*-hydroxybenzyl alcohol. It is therefore assumed that this glucoside and compound (XIII) are *p*-hydroxybenzyl β-glucoside.

A compound (XIV) with $\lambda_{max.}$ 294 mμ was found in extracts of beans that had been fed with *p*-nitrophenol. The compound was electrophoretically immobile with all the buffers used, and could be detected on paper with a spray reagent for nitro compounds (Maddy, 1959). This evidence, together with hydrolysis studies, suggests that (XIV) is *p*-nitrophenyl β-glucoside. 2,4-Dinitrophenol-treated seeds did not produce detectable glucosides.

Glucosylation by wheat-germ and bean enzymes with uridine diphosphate glucose and other donors

A wheat-germ enzyme (cf. Yamaha & Cardini, 1960*a*) with UDP-glucose produced the corresponding mono β-glucosides when incubated with quinol, resorcinol and catechol. With saligenin, and *m*-hydroxybenzyl alcohol, only the alcoholic groups were glucosylated, giving *o*-hydroxy-(IX) and *m*-hydroxy-(XI)-benzyl β-glucoside. No glucoside formation could be detected with *p*-hydroxybenzyl alcohol. Pyrogallol gave one glucoside that was identical with 2,3-dihydroxyphenyl β-glucoside (IV); 2,4-dihydroxyphenyl β-glucoside (VI) was the only compound obtained from 1,2,4-trihydroxybenzene. When UDP-glucose was replaced by potential glucose donors such as α-D-glucose 1-phosphate, maltose, cellobiose or methyl α-D-glucoside, glucose transfer could not be demonstrated with resorcinol as an acceptor. When ATP, UTP and α-D-glucose 1-phosphate were used to replace UDP-glucose, however, mono-β-glucoside formation was observed with quinol, resorcinol, catechol and saligenin as acceptor substrates. (With the last-named, *o*-hydroxybenzyl β-glucoside, IX, was again formed.) Attempts to synthesize phenolic galactosides by replacing α-D-glucose 1-phosphate by α-D-galactose 1-phosphate failed. A phosphate buffer extract of dormant broad-bean seeds also glucosylated quinol, resorcinol, catechol and saligenin in the presence of UDP-glucose, giving the same products as the wheat-germ enzyme. This preparation was, however, less active than the wheat-germ enzyme.

Experiments with emulsin and invertase

Incubation of quinol, resorcinol and 1,2,4-trihydroxybenzene with cellobiose, arbutin, methyl β-D-glucoside or high concentrations of D-glucose in the presence of β-glucosidase (cf. Bourquelot & Hérissey, 1913) did not produce glucosides, and fructose could not be transferred from sucrose to quinol or 1,2,4-trihydroxybenzene by yeast invertase. This latter enzyme did, however, produce a fructoside (XV) with saligenin, which gave a pink colour with the diazonium spray *A*, thus indicating the presence of a free phenolic hydroxyl group. This was supported by the spectrum, $\lambda_{max.}$ 274 mμ, changing to 291 mμ with alkali. The compound could also be located on chromatograms as a blue spot [R_F 0·68 in solvent *A*, cf. compound (IX), Table 1] with urea hydrochloride, a specific reagent

for ketoses, and on hydrolysis with yeast invertase it yielded saligenin and fructose (the compound was not hydrolysed by β-glucosidase). These hydrolysis products were also rapidly released when (XV) was heated with 0·01 N-sulphuric acid (*o*-hydroxybenzyl β-glucoside is stable under these conditions). This evidence strongly suggests that (XV) is *o*-hydroxybenzyl β-fructofuranoside.

DISCUSSION

This study has shown that the major primary products formed by feeding broad-bean seeds with mono-, di- and tri-hydric phenols are the corresponding mono-β-glucosides. The formation of oligoglucosides or glucosylation of more than one hydroxyl group cannot be altogether excluded in view of the difficulty in detecting small amounts of these higher-molecular-weight compounds in the complex extracts.

With enzyme preparations from wheat-germ and broad-bean seeds with UDP-glucose as the glucose donor, phenols were also glucosylated *in vitro* and the resulting products closely resembled those obtained from the experiments *in vivo*. The reaction with the di- and tri-hydric phenols *in vitro* did, however, appear to be more specific and only one isomer in each case resulted, this corresponding to the major component produced in the feeding experiments. Glucosylation of phenols has also been achieved with UTP, α-D-glucose 1-phosphate and a wheat-germ extract, i.e. utilizing the UDP-glucose-pyrophosphorylase of the germ. Attempts to synthesize phenolic glucosides by simple transferase reactions involving almond β-glucosidase and 'low energy'-potential glucose donors failed. Similar unsuccessful experiments have been recorded in the past with glucose-containing disaccharides, aryl- and alkyl-glucosides and glucose as donors (Cardini & Yamaha, 1958; Pridham, 1960*b*; Anderson *et al.* 1960). Such systems will, of course, glucosylate aromatic and aliphatic alcohols (Rabaté, 1935; Anderson *et al.* 1960; Dedonder, 1961; Jermyn, 1961). Fructosylation of phenolic hydroxyl groups also could not be effected with sucrose and yeast invertase although here again this enzyme readily transfers fructose to other sugar molecules and to simple aliphatic alcohols (Edelman, 1956; Dedonder, 1961) and now, as we have shown, to saligenin, forming *o*-hydroxybenzyl β-fructofuranoside.

UDP-glucose occurs widely in plant tissues (Ginsburg, Stumpf & Hassid, 1956; Rowan, 1959; Ziegler, 1960; Dutton, Carruthers & Oldfield, 1961), and Abdel-Wahab & El-Kinawi (1959, 1960) have presented evidence which suggests that it occurs in *Vicia faba*. We also have chromatographic evidence for the existence of this nucleotide in the broad bean. It is probable therefore that UDP-glucose is a donor molecule for the glucosylation of phenols in the bean, and indeed in all higher plant tissues although other nucleotide derivatives, such as thymidine diphosphate glucose (Barber, 1962), may also be involved in this reaction.

Many different types of phenols can be glucosylated by plant tissues. This may be due to the presence in the cells of a number of different enzymes (or different active sites on one enzyme) with relatively high acceptor specificities or to a single enzyme of low specificity. The former hypothesis at present seems more likely in view of the isolation from wheat germ of an enzyme which is specific for the glucosylation of phenols with 1,4-dihydroxy groupings. Crude wheat-germ extracts with UDP-glucose will glucosylate phenols with other hydroxyl arrangements (Yamaha & Cardini, 1960*a*). A multi-enzyme system in which the components have different activities could also account for the different amounts of the isomeric monoglucosides produced on feeding di- and tri-hydric phenols and phenolic alcohols to bean seeds. With a single enzyme these results might be explained by stereospecificity or by the relative chemical reactivity of hydroxyl groups in the polyhydric compound. Hydroxyl reactivity and stereospecificity would presumably also play important roles with a multi-enzyme system. Preliminary feeding experiments with pyrogallol, saligenin and *p*-hydroxybenzyl alcohol first led us to believe that the most strongly dissociated hydroxyl group was most readily glucosylated but this theory became unsatisfactory when a number of other phenols were tested *in vivo* and *in vitro*. (With saligenin, hydrogen-bond formation between the hydrogen of the phenolic hydroxyl group and the alcoholic oxygen atom strongly activates the alcohol group, and one would expect this on purely chemical grounds to be readily glucosylated, as was the case.)

It should also be remembered that results from feeding experiments and experiments with crude enzymes *in vitro* could be misleading owing to the instability of the phenolic glucosides to other enzyme systems such as the hydrolases and phenol oxidases.

It is of interest to consider the biosynthesis of salicin as our results suggest that saligenin is not the precursor. Ciamician & Ravenna (1916) claimed that salicin was formed by maize seedlings that had been treated with saligenin. Our own experiments with maize and *Salix daphnoides* strongly suggest that *o*-hydroxybenzyl β-glucoside is the major product formed from saligenin. With maize, no salicin could be detected. Salicin occurs naturally in *Salix daphnoides*, but here no marked increase was observed. These differences in results may be due to the fact that Ciamician & Ravenna (1916)

used a method that did not clearly distinguish between the two isomeric glucosides of saligenin. According to Yamaha & Cardini (1960*a*) wheat-germ extracts incubated with UDP-glucose and saligenin produce a compound with similar chromatographic properties to salicin. Here again our own experiments with wheat-germ and broad-bean enzymes showed clearly that the major product is the isomeric glucoside and that little, if any, salicin is produced. It is possible therefore that *in vivo* saligenin is not the precursor of salicin, always assuming that our feeding experiments allowed the phenol to reach the correct site for salicin synthesis in the plant. Ibrahim & Towers (1959) suggest that salicylic acid may be a common metabolite of plants, and Klämbt (1962) has shown that benzoic acid can be converted into salicylic acid and salicylic acid β-glucoside by several species of plants. It is conceivable therefore that salicin biosynthesis proceeds via the corresponding glucoside followed by reduction of the carboxyl group to a primary alcohol group. In this connexion, it can be noted that helicin (*o*-formylphenyl β-D-glucoside), gaultherin (*o*-carboxymethylphenyl 6-*O*-β-D-xylosyl-β-D-glucoside) and violutin (*o*-carboxymethylphenyl 6-*O*-β-L-arabinosyl-β-D-glucoside) occur naturally in plants (McIlroy, 1951); a glycoside of salicylic acid has so far not been found, however.

SUMMARY

1. The structures of the mono β-glucosides formed by germinating broad-bean seeds in the presence of various phenols have been studied.

2. Enzyme preparations from wheat-germ and broad-bean seeds will glucosylate phenolic and alcoholic hydroxyl groups in the presence of uridine diphosphate glucose (or for wheat-germ enzyme, with uridine triphosphate and α-D-glucose 1-phosphate). The products obtained with these enzymes closely resemble those produced in the experiments *in vivo*.

3. The nature of the phenol-glucosylating system in plants is considered.

4. Saligenin *in vivo* and *in vitro* mainly gives rise to *o*-hydroxybenzyl β-glucoside, and not salicin. The biosynthesis of this latter compound is discussed.

We are greatly indebted to Spillers Ltd. for a generous gift of wheat germ and to Professor E. Adler for specimens of methylated phenols. The paper-electrophoresis apparatus was built with the aid of a grant from The Royal Society. Professor E. J. Bourne is thanked for his interest and encouragement and M.J.S. acknowledges the receipt of a postgraduate studentship from the Department of Scientific and Industrial Research.

REFERENCES

Abdel-Wahab, M. F. & El-Kinawi, S. A. (1959). *Acta chem. scand.* **13**, 1653.
Abdel-Wahab, M. F. & El-Kinawi, S. A. (1960). *Acta chem. scand.* **14**, 1667.
Anderson, J. D., Hough, L. & Pridham, J. B. (1960). *Biochem. J.* **77**, 564.
Barber, G. A. (1962). *Biochemistry*, **1**, 463.
Bourquelot, E. & Hérissey, H. (1913). *C.R. Acad. Sci., Paris*, **156**, 1790.
Cardini, C. E. & Leloir, L. F. (1957). *Cienc. e Invest.* **13**, 514.
Cardini, C. E. & Yamaha, T. (1958). *Nature, Lond.*, **182**, 1446.
Ciamician, G. L. & Ravenna, C. (1916). *R.C. Accad. Lincei*, **18**, 419.
Dedonder, R. A. (1961). *Annu. Rev. Biochem.* **30**, 347.
Dutton, J. V., Carruthers, A. & Oldfield, J. F. T. (1961). *Biochem. J.* **81**, 266.
Edelman, J. (1956). *Advanc. Enzymol.* **17**, 189.
Gallent, J. B. (1958). *J. org. Chem.* **23**, 75.
Ginsburg, V., Stumpf, P. K. & Hassid, W. Z. (1956). *J. biol. Chem.* **223**, 977.
Harborne, J. B. & Corner, J. J. (1961). *Biochem. J.* **81**, 242.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Ibrahim, R. K. & Towers, G. H. N. (1959). *Nature, Lond.*, **184**, 1803.
Isherwood, F. A. (1954). *Brit. med. Bull.* **10**, 202.
Jermyn, M. A. (1961). *Rev. pure appl. Chem.* **11**, 92.
Klämbt, H. D. (1962). *Nature, Lond.*, **196**, 491.
Kosuge, T. & Conn, E. E. (1959). *J. biol. Chem.* **234**, 2133.
McIlroy, R. J. (1951). *The Plant Glycosides*, p. 15. London: Edward Arnold and Co.
Maddy, A. H. (1959). *Nature, Lond.*, **184**, 1397.
Mansfield, G. H., Swain, T. & Nordstrom, C. G. (1953). *Nature, Lond.*, **172**, 23.
Marsh, C. A. (1960). *Biochim. biophys. Acta*, **44**, 359.
Nierenstein, M. (1930). *J. Amer. chem. Soc.* **52**, 4012.
Pridham, J. B. (1957). *Analyt. Chem.* **29**, 1167.
Pridham, J. B. (1959). *J. Chromat.* **2**, 605.
Pridham, J. B. (1960*a*). In *Phenolics in Plants in Health and Disease*, p. 9. Ed. by Pridham, J. B. London: Pergamon Press Ltd.
Pridham, J. B. (1960*b*). *Biochem. J.* **76**, 13.
Pridham, J. B. (1961). *Chem. & Ind.* p. 1172.
Pridham, J. B. & Saltmarsh, M. J. (1960). *Biochem. J.* **74**, 42P.
Pridham, J. B. & Saltmarsh, M. J. (1962). *Biochem. J.* **82**, 44P.
Rabaté, J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
Rabaté, J. & Ramart-Lucas, P. (1935). *Bull. Soc. chim. Fr.* **2**, 1596.
Rowan, K. S. (1959). *Biochim. biophys. Acta*, **34**, 270.
Swain, T. (1953). *Biochem. J.* **53**, 200.
Swain, T. (1954). *Chem. & Ind.* p. 1480.
Trivelloni, J. C., Recondo, E. & Cardini, C. E. (1962). *Nature, Lond.*, **195**, 1202.
Wickberg, B. (1958). *Acta chem. scand.* **12**, 615.
Yamaha, T. & Cardini, C. E. (1960*a*). *Arch. Biochem. Biophys.* **86**, 127.
Yamaha, T. & Cardini, C. E. (1960*b*). *Arch. Biochem. Biophys.* **86**, 133.
Ziegler, H. (1960). *Naturwissenschaften*, **47**, 140.

B12

[*Reprinted from the Proceedings of the Biochemical Society*, 16–17 *May* 1963.
Biochem. J., 1963, Vol. 88, No. 2, 45–46P.]

Deamination of β-(3,4-Dihydroxyphenyl)-L-alanine by Plants

By NORMA J. MACLEOD and J. B. PRIDHAM.
[*Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey*]

Neish (1961) has isolated an enzyme, tyrase, from monocotyledons which deaminates L-tyrosine with the formation of *trans-p*-coumaric acid. An ammonia-lyase has also been found in monocotyledons and dicotyledons which converts L-phenylalanine into *trans*-cinnamic acid (Koukol & Conn, 1961).

We have recently shown that buffered extracts of acetone powders from dandelion (*Taraxacum officinale*) leaves possess β-(3,4-dihydroxyphenyl)-L-alanine (DOPA) ammonia-lyase activity. The *trans*-caffeic acid produced from DOPA by this enzyme has been characterized by extensive paper chromatographic and electrophoretic studies, ultraviolet spectrophotometry and by catalytic hydrogenation to β-(3,4-dihydroxyphenyl)propionic acid. The optimum pH for the enzyme reaction is 8·8.

The DOPA ammonia-lyase activities of several plant species have been compared. The activity in barley (*Hordeum vulgare*) and dandelion is relatively high, whereas in potato (*Solanum tuberosum*) tuber tissue it is negligible. The activity in broad-bean (*Vicia faba*) tissues is also low and it is suggested that this may, at least in part, be related to the high concentration of DOPA found in this plant.

We are indebted to Professor E. J. Bourne and Drs E. C. Bate-Smith and T. Swain for their interest and to the Agricultural Research Council and the United States Department of Agriculture for financial assistance.

Koukol, J. & Conn, E. E. (1961). *J. biol. Chem.* **236**, 2692.
Neish, A. C. (1961). *Phytochem.* **1**, 1.

B13

Reprinted from

PHYTOCHEMISTRY

Vol. 3, No. 4

PERGAMON PRESS

OXFORD · LONDON · NEW YORK · PARIS

Phytochemistry, 1964, Vol. 3, pp. 493 to 497. Pergamon Press Ltd. Printed in England

THE PHENOL GLUCOSYLATION REACTION IN THE PLANT KINGDOM

J. B. PRIDHAM

Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 5 *February* 1964)

Abstract—The ability of various plant tissues to convert quinol and resorcinol to the corresponding mono-β-D-glucopyranosides *in vivo* has been studied. This activity is prominent in the angiosperms (except for some water plants) and gymnosperms and appears to be entirely absent in the algae and fungi. The significance of these findings is discussed.

INTRODUCTION

SEVERAL workers have clearly demonstrated that foreign phenols when introduced into the leaves and seedlings of higher flowering plants are rapidly converted to mono-β-D-glucopyranoside derivatives.[1-9] The D-glucose donor for these reactions *in vivo* is almost certainly a nucleoside pyrophosphate derivative, probably uridine diphosphoglucose (UDPG), the reaction proceeding as follows:

$$\text{UDPG} + \text{Ar. OH} \xrightarrow{\text{UDPG: phenol glucosyltransferase}} \text{Ar. OG} + \text{UDP}$$

Evidence in favour of such a reaction occurring *in vivo* is:

(1) glucosylation of phenols can be effected *in vitro* using UDPG and plant extracts;[7,10-12]
(2) UDPG would appear to be ubiquitous in higher plants;[13]
(3) a reaction involving a 'high energy' glucose donor would be energetically favourable. All attempts to carry out enzymic synthesis of *O*-β-D-glucopyranosides of phenols using "low energy" glucose donors (e.g. α-D-glucose-1-phosphate or glucose disaccharides) and plant enzymes have, so far, failed.[14]

[1] G. CIAMICIAN and C. RAVENNA, *Atti reale accad. Lincei*, **25**, 3 (1916).
[2] T. MIWA, S. NAKAMURA and A. SHIBATA, *Kôso Kagaku Shinpojiuma*, **12**, 48 (1957); *Chem. Abstracts*, **52**, 1314 (1958).
[3] A. HUTCHINSON, C. ROY and G. H. N. TOWERS, *Nature*, **181**, 841 (1958).
[4] J. B. PRIDHAM, *Nature*, **182**, 795 (1958).
[5] C. W. NYSTROM, N. E. TOLBERT and S. H. WENDER, *Plant Physiol.* **34**, 142 (1959).
[6] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **234**, 2133 (1959).
[7] J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **74**, 42P (1960); *Biochem. J.* **87**, 218 (1963).
[8] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).
[9] V. C. RUNECKLES and K. WOOLRICH, *Phytochemistry*, **2**, 1 (1963).
[10] T. YAMAHA and C. E. CARDINI, *Biochem. Biophys. Acta*, **86**, 127 (1960).
[11] G. J. DUTTON and A. M. DUNCAN, *Biochem. J.* **77**, 18P (1960).
[12] G. A. BARBER, *Biochem.* **1**, 463 (1962).
[13] Occurrence of UDPG briefly reviewed in ref. 7.
[14] C. E. CARDINI and T. YAMAHA, *Nature*, **182**, 1446 (1958). J. B. PRIDHAM, *Biochem. J.* **76**, 13 (1960). J. D. ANDERSON, L. HOUGH and J. B. PRIDHAM, *Biochem. J.* **77**, 564 (1960).

The following investigation was mainly undertaken in order to discover whether lower plants could glucosylate phenolic hydroxyl groups and also to survey the occurrence of this activity in gymnosperms and a wide variety of angiosperms.

RESULTS AND DISCUSSION

Table 1 shows clearly that the ability to glucosylate phenols is possessed by most angiosperms and that it is not confined to leaves and developing seedlings. Within this group,

TABLE 1. FORMATION OF β-D-GLUCOPYRANOSIDES OF QUINOL AND RESORCINOL BY PLANTS

Plant	Organ or tissue tested	Glucoside formed from*	
		Quinol	Resorcinol
Angiosperms			
Arrhenatherum elatius (L.) Beauv. ex J. & C. Presl.	leaf		+
Castanea sativa Mill.	leaf	+ +	+ +
Citrus sirensis Osbeck	leaf	+ +	+ +
Cotyledon paraguayense E. Walther	stem slices	+ +	+ +
Cotyledon sp.	leaf slices	+	+
Darlingtonia californica Torr.	leaf		+ +
Deschampsia flexuosa (L.) Trin.	leaf	+ +	+ +
Drosera capensis L.	leaf	+	+
Elodea canadensis Michx.	whole organism	−	−
Ficus elastica Roxb.	leaf	+ +	+ +
Ilex aquifolium L.	leaf	+ +	+ +
Iris germanica L.	leaf	+ +	+ +
Lemna minor L.	whole organism	−	−
Narcissus jonquilla L.	leaf	+	+
Nepenthes hookeriana Lindl.	leaf	+ +	+ +
Nymphaea alba L.	shoot	+ +	+ +
Paphiopedilum insigne Pfitz.	leaf	+ +	+ +
Polygonatum multiflorum (L.) All.	rhizome	+	+ +
Quercus robur L.	leaf	+ +	+ +
Solanum tuberosum L.	tuber slices	+	+
Taraxacum officinale Weber	leaf	+ +	+ +
	floret	+	+
	tap root	+	+
Utricularia vulgaris L.	whole organism	−	−
Viscum album L.	leaf	+ +	+ +
Gymnosperms			
Cycas revoluta Thunb.	leaf	+ +	+ +
Cupressus lawsoniana A. Murray	leaf	+ +	+ +
Ginkgo biloba L.	leaf	+ +	+ +
Larix (Mill.) sp.	leaf	+ +	+ +
Pinus radiata D. Don	leaf	+ +	+ +
Ferns			
Equisitum arvense L.	branch	+ +	+ +
Polypodium (L.) sp.	leaf	+	+
Polystichum setiferum (Forsk.) Woynar	leaf	+	+
Mosses			
Acrocladium cuspidatum (Hedw.) Lindb.	shoot	±	±
Atrichum undulatum (Hedw.) P. Beauv.	shoot	+	+
Brachythecium rutabulum (Hedw.) B. & S.	shoot	−	−
Cratoneuron commutatum (Hedw.) Roth.	shoot	−	−
Ctenidium molluscum (Hedw.) Mitt.	shoot	±	±

TABLE 1 (continued)

Plant	Organ or tissue tested	Glucoside formed from*	
		Quinol	Resorcinol
Drepanocladus fluitans (Hedw.) warnst.	shoot	±	±
Funaria hygrometrica Hedw.	shoot		+
Leucobryum glaucum (Hedw.) Schp.	shoot	±	±
Philonatis fontana (Hedw.) Brid.	shoot	+	+
Pseudoscleropodium purum (Hedw.) Fleisch.	shoot	−	−
Sphagnum papillosum Lindb.	shoot	±	±
Liverwort			
Marchantia polymorpha L.	thallus	+	+
Algae			
Ascophyllum nodosum Le Jol.	thallus	−	−
Dilsea edulis Stackhouse	thallus	−	−
Fucus serratus L.	thallus	−	−
Fucus vesiculosus L.	thallus	−	−
Gigatina stellata Batt.	thallus	−	−
Halidrys siliquosa Lyngb.	thallus	−	−
Laminaria digitata Lamouroux	thallus	−	−
Pelvetia caniculata Decaisne & Thuret	thallus	−	−
Ulva lactuca L.	thallus	−	−
Uronema gigas Vischer	whole cells	−	−
Fungi			
Aspergillus niger van Tiegh.	mycelium	−	−
Psalliota campestris (Fr.) Quel.	fruiting body	−	−

*−, no glucoside detected; ±, traces present; +, small amounts; ++, high concentrations formed.

plants giving a positive reaction included monocotyledenous and both woody and herbaceous dicotyledenous species some of which are succulents or parasites. The results with three species of water plants, however, were unexpectedly negative. Neither *Utricularia vulgaris* nor *Lemna minor* could under any circumstances be induced to form glucosides; with *Elodea canadensis* glucosylation was doubtful, although traces may have been formed when resorcinol was fed in 0·25% concentration. Attempts to synthesize *m*-hydroxyphenyl-β-D-glucopyranoside using an enzyme preparation from *Lemna* with UDPG and resorcinol also failed.

All the gymnosperms that were tested also converted quinol and resorcinol to the corresponding mono-β-D-glucosides, and the activities exhibited by the leaves of these species appeared to be as high as those of the angiosperms. The species of ferns that were examined were also able to form glucosides but, in general, the activity appeared to be somewhat lower than that of the flowering plants. Some activity was present in most of the mosses that were tested but was extremely low in the majority of cases. Incubating an acetone powder prepared from *Cratoneuron commutatum* with UDPG and resorcinol produced no glucoside.

No glucosylation reactions were observed with fungi or with algae from fresh or salt water habitats, but the liverwort, *Marchantia polymorpha*, did exhibit definite activity. These latter results confirm the findings of Roy[15] who was unable to demonstrate the formation of phlorin (3,5-dihydroxyphenyl-β-D-glucopyranoside) when species of *Ulva*, *Fucus*, *Laminaria*, *Agardhiella* and *Polysiphonia* were treated with phloroglucinol and ^{14}C-labelled glucose. *Marchantia* produced a small amount of phlorin under similar conditions (cf.[3]).

It would appear from the results in Table 1 that a marked ability to glucosylate phenols is

[15] C. ROY, M.Sc. Thesis, McGill University, Montreal, Canada (1959).

characteristic of the majority of higher plants, but that this reaction is absent or occurs at a very slow rate in Bryophytes and Thallophytes. The failure of a particular species to glucosylate quinol or resorcinol can presumably be accounted for by the absence of the necessary enzyme and/or the glucose donor. There is every reason to suppose that nucleoside diphosphoglucose derivatives are common constituents of all plant life and therefore the inability to form glucosides is probably due to an enzyme deficiency. This is certainly borne out by the present *in-vitro* experiments with acetone powders from *Lemna* and *Cratoneuron*. Other factors which could interfere with studies of this kind using whole organisms or plant organs are: (1) poor uptake of the phenol by the tissue and (2) inhibition of the enzyme system by the phenol. In this particular study no difficulties in uptake were apparent, and where species exhibited an initial negative activity, the experiment was repeated using lower concentrations of the phenol in order to try and obviate possible toxic effects.

If, as has often been suggested, glucosylation serves as one method for the detoxification of harmful phenolic compounds which could either arise from normal plant metabolism or from the environment,[16] it would be interesting to know if the lower forms of plant life have other methods for dealing with these compounds. For example, this study with species of *Aspergillus* and *Psalliota* and investigations by Towers[17] and Roy[15] with *Aspergillus giganteus* Wehmer and species of *Penicillium* and *Rhizopus* suggest that fungi are unable to transfer glucosyl residues to phenols *in vivo*, and it is probable that detoxification of these compounds by fungi occurs by oxidative processes.[18] On the other hand, can the seaweeds, which are rich in sulphated polysaccharides, detoxify phenols by a sulphation process similar to that occurring in animal tissues?[19]

Reports of phenolic glucosides or other glycosides occurring naturally in many of the lower groups of plants are very few in number although it must be stressed that detailed investigations of these plants has not so far been carried out. It would be also interesting to know whether the activity of the enzyme system responsible for the glucosylation of quinol and resorcinol is related to the concentration of naturally occurring phenolic glucosides in the tissue. It should not necessarily be assumed that such a relationship exists, since enzymes specific for the glucosylation of phenols other than quinol and resorcinol may be present.

It would also be useful to investigate further examples of fresh-water angiosperms and algae. In the former group only the free-floating plants were inactive; *Nymphaea*, which has a root, showed a normal angiosperm activity. Further comparative work along these lines might yield results of taxonomic interest.

MATERIALS AND METHODS

Plant specimens were obtained from the Botany Department and grounds of Royal Holloway College and the University of London Botanical Supply Unit.

Feeding experiments were carried out at room temperature for periods of 12–24 hr using 1% aqueous solutions of quinol and resorcinol. The cut ends of the various plant organs that were examined were placed in the phenol solutions. *Elodea*, *Utricularia*, the algae and all tissue slices were completely immersed in aerated solutions and *Lemna* was allowed to float on the surface of solutions. In all cases control experiments, replacing phenol solutions with

[16] Reviewed by J. B. PRIDHAM in *Phenolics in Plants in Health and Disease* (Ed. J. B. PRIDHAM), p. 9, Pergamon Press, London (1960).
[17] G. H. N. TOWERS, unpublished results.
[18] D. WOODCOCK in ref. 16, p. 75.
[19] R. T. WILLIAMS, *Detoxication Mechanisms*, Wiley, New York (1947).

water, were carried out. After the required feeding period the tissues were carefully washed with water, blotted dry, and then macerated with hot 70% aqueous methanol. The extracts were filtered, concentrated under reduced pressure at 40° and then examined on paper chromatograms (butan-1-ol–ethanol–water 40:11:19, by volume; diazotized *p*-nitroaniline/NaOH spray reagent[20]) together with authentic specimens of arbutin (*p*-hydroxyphenyl-β-D-glucopyranoside) and *m*-hydroxyphenyl-β-D-glucopyranoside. These standard compounds give characteristic, stable azo dyes with the diazonium reagent which under alkaline conditions are blue and red, respectively. Species which gave negative results under these conditions were re-examined using lower phenol concentrations (0·5 and 0·25% w/v). Penetration of the tissues by the phenols was in all cases indicated by the presence of relatively large amounts of the free compounds in the methanolic extracts.

Acetone powders of *Lemna* and *Cratoneuron* were prepared and extracted, separately, with 0·05 M-Tris-HCl buffer (pH 7·2) containing 0·5% L-cysteine (ca. 1 g acetone powder to 50 ml buffer). These extracts were then incubated at 35° with resorcinol and UDPG using the conditions described by Pridham and Saltmarsh.[7] Control digests omitting the UDPG were also set up. Paper chromatographic examination, using the solvent and spray reagent given above, showed that no *m*-hydroxyphenyl-β-D-glucopyranoside was formed in either case.

Acknowledgements—The author is indebted to Drs. J. Evans and E. Lodge for helpful discussions.

[20] T. SWAIN, *Biochem. J.* **53**, 200 (1953).

B14

(*Reprinted from Nature, Vol. 202, No. 4931, pp. 488–489, May 2, 1964*)

Enzymatic Galactosylation and Fucosylation of Phenols

PHENOLIC β-D-glucopyranosides have been synthesized by incubating phenols with plant enzymes in the presence of uridine diphosphate glucose[1–5]. All attempts to form β-D-glucosides by the enzymatic transfer of D-glucose from 'low-energy' donors (such as disaccharides, α-D-glucose-1-phosphate and simple β-D-glucosides) have so far failed using higher plants as the source of enzymes[2,6–8]. β-D-Glucosides of resorcinol and other phenols have, however, been prepared from maltose (or isomaltose), as a source of glucose, and an intracellular enzyme preparation from *Aspergillus niger*[9].

We now wish to report the enzymatic synthesis of phenolic β-D-galactosides and β-D-fucosides using *E. coli* β-galactosidase[10].

Lactose (180 μM), resorcinol (140 μM), β-galactosidase (0·02 ml.; $1{\cdot}6 \times 10^5$ units) in M/15-sodium phosphate buffer (*p*H 7·0; 1·0 ml.) were incubated together at 30° and aliquots withdrawn at intervals up to 1 h and examined on paper chromatograms (butan 1-ol/ethanol/water (40 : 11 : 19, by vol.), solvent *A*; diazotized *p*-nitroaniline/sodium hydroxide spray reagent[11]). After incubation for 3 min a compound (I; R_F 0·53) giving an intense pink spot was detected on the chromatogram. At 15 min two slower moving compounds (R_F 0·31 and 0·41) giving pink colorations were also discernible. The reaction was stopped after 60 min by heating the remaining mixture at 80° for 5 min. Compound (I) was isolated by chromatography on thick paper (solvent *A*) and characterized by further paper chromatography, paper electrophoresis, ultra-violet spectroscopy and hydrolysis with β-galactosidase. The results are given in Table 1 together with those for authentic *m*-hydroxyphenyl-β-D-glucopyranoside, for comparative purposes.

The experiment was repeated using sodium phosphate buffer (0·75 ml.) and *o*-nitrophenyl-β-D-fucopyranoside solution (0·5 ml. saturated solution in phosphate buffer) in place of lactose. The reaction was again followed by paper chromatography and this showed the gradual formation of a compound (II) (R_F 0·68; solvent *A*) which gave the same pink colour with the diazonium spray reagent as compound (I). Compound (II) was isolated and examined by the same methods used with (I) (Table 1).

The results show clearly that (I) and (II) are *m*-hydroxyphenyl-β-D-galactoside and *m*-hydroxyphenyl-β-D-fucoside,

Table 1. PROPERTIES OF RESORCINOL GLYCOSIDES

Compound	R_F		*MSA** 0·1 M sodium borate buffer (*p*H 10·0)	λ_{max}		β-Galactosidase hydrolysis products‡
	Solvent *A*	Solvent *B*†		in H_2O	in NaOH	
m-Hydroxyphenyl-β-D-glucopyranoside	0·56	0·49	0·50	271	285	—
I	0·53	0·46	0·68	270	285	Resorcinol, galactose
II	0·68	0·64	0·62	270	285	Resorcinol, fucose

* Electrophoretic mobility relative to salicyclic acid.
† *B*, ethylacetate/acetic acid/water (9 : 2 : 2, by vol.).
‡ Hydrolysis products detected on paper chromatograms. Sugars, solvent *B* (silver nitrate/sodium hydroxide spray reagent); resorcinol, solvent *A* (diazotized *p*-nitroaniline/sodium hydroxide spray reagent).

respectively, both glycosyl groups presumably occurring in the stable pyranoid form. The two higher molecular weight compounds produced from lactose and resorcinol were probably *O*-digalactosyl derivatives of the phenol.

One of us (J. B. P.) thanks the Department of Chemistry, University of Freiburg, for supplying facilities.

J. B. PRIDHAM

Department of Chemistry,
Royal Holloway College (University of London),
Englefield Green, Surrey.

K. WALLENFELS

Chemisches Laboratorium der Universität,
Freiburg i. Br., Germany.

[1] Cardini, C. E., and Leloir, L. F., *Cienc. Invest.*, **13**, 514 (1957).
[2] Pridham, J. B., and Saltmarsh, M. J., *Biochem. J.*, **74**, 42 P (1960); **87**, 218 (1963).
[3] Yamaha, T., and Cardini, C. E., *Arch. Biochem. Biophys.*, **86**, 127 (1960).
[4] Dutton, G. J., and Duncan, A. M., *Biochem. J.*, **77**, 18 P (1960).
[5] Barber, G. A., *Biochem.*, **1**, 463 (1962).
[6] Cardini, C. E., and Yamaha, T., *Nature*, **182**, 1446 (1958).
[7] Anderson, J. D., Hough, L., and Pridham, J. B., *Biochem. J.*, **77**, 564 (1960).
[8] Pridham, J. B., *Biochem. J.*, **76**, 13 (1960).
[9] Pridham, J. B., *Chem. and Indust.*, 1172 (1961).
[10] Wallenfels, K., Zarnitz, M. L., Laule, G., Bender, H., and Keser, M., *Biochem. Z.*, 331, 459 (1959).
[11] Swain, T., *Biochem. J.*, **53**, 200 (1953).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

B15

Reprinted from
"METHODS IN POLYPHENOL CHEMISTRY"
Proceedings of the Plant Phenolics Group Symposium
Oxford, April 1963
PERGAMON PRESS
OXFORD · LONDON · EDINBURGH · NEW YORK · PARIS · FRANKFURT
1964

CHAPTER 9

PAPER ELECTROPHORESIS OF PHENOLIC COMPOUNDS

J. B. PRIDHAM

Department of Chemistry, Royal Holloway College, University of London

PAPER electrophoresis has not been used so widely as paper chromatography for the examination of phenols. This is probably due to the fact that chromatographic techniques are simple and generally quite adequate for analysis and fractionation of these compounds. In addition, the main advantage in using paper electrophoresis is that rapid separations can be achieved by the use of high potential gradients and this requires a somewhat costly apparatus.

Paper electrophoresis is not a substitute for paper chromatography but serves as a very useful complementary technique. It is perhaps most useful for qualitative analysis by direct comparison with known compounds but it may also be employed for the fractionation of complex mixtures of phenols (e.g. the betacyanins)[1, 2] and for the elucidation of certain structural features present in phenolics. For preparative purposes continuous paper electrophoresis[3] or column electrophoresis with cellulose powder[4, 5] may be used.

The theoretical explanations of the relative rates of movement of phenols on paper electrophoretograms is not nearly so advanced as in the case of the carbohydrates (see Chapter 8) and again, in comparison with carbohydrates, there is little scope for complex formation except with *o*-dihydroxyl groups. Both of these factors mean that, at the present time, the method can only be used for the detection of a very limited number of structural features present in phenols.

Franc and Wurst[6] have investigated the contribution to the absolute mobility of aromatic compounds made by a number of functional groups. These workers have constructed a table (Table 1) of group contributions which can be used to calculate the absolute mobilities of derivatives of benzene and naphthalene with a fair degree of accuracy. In general, the contributions are additive and the relative positions of the functional groups on the aromatic nucleus have little effect on the mobility. In the case of *ortho*-substituents which are hydrogen bonded, however, correction factors have to be included. Resonance effects can introduce a serious disagreement between observed and calculated mobilities and it is doubtful whether Franc and Wurst's rules could be applied to complex polyphenols.

TABLE 1. RELATIONSHIP BETWEEN STRUCTURES OF AROMATIC COMPOUNDS AND ELECTROPHORETIC MOBILITIES USING ALKALINE AND ACIDIC ELECTROLYTES[6]

Contributions of functional groups to total mobility ($u \times 10^5$ cm^2 V^{-1} sec^{-1})	In 3 N NH_4OH	In N CH_3CO_2H
—OH	11·3	0
2—OH	20·4	0
3—OH	28·1	—
—CO_2H	11·3	1·6
2—CO_2H	20·4	3·2
—SO_3H	10·0	7·1
2—SO_3H	21·1	13·0
3—SO_3H	27·3	17·2
—NH_2	−0·7	−7·1
2—NH_2	−1·4	−13·4
—NO_2	0	0
—CH_3	0	0
—CHO	0	1·2
—Cl	0	0
—OCH_3	0	0
Correction factors for H-bonding		
—O—H- - - -O—	−5·9	3·1
—NO_2 · · · · H—O— (Compound also possessing *p*-nitro group)	−1·6	0
—NO_2- - - -H—O—	1·2	0
—NO_2- - -H—N— (Compound also possessing *p*-nitro group)	−0·8	3·2
—O- - -H—N—	1·7	3·2

A phenolic compound must, of course, carry a charge in order to exhibit electrophoretic mobility. This charge may result from the ionization of the phenolic hydroxyl group(s) or other functional groups which are present in the molecule, such as carboxyl or amino. An increased charge may be conferred on the phenol by complex formation (which usually involves inorganic anions and *o*-dihydroxyl groups) or by the formation of derivatives such as acidic azo dyes or, in the case of phenolic aldehydes, hydroxysulphonic acids.

In addition to the charge carried by the molecule, the rate of movement may also be influenced by molecular weight, stability and nature of the complex and adsorption and solubility factors. Streaking (tailing) of the spots on the paper may result from adsorption or oxidation or, in the case of electrolytes containing complexing ions, by the formation of more than one complex.[7]

At Royal Holloway College one of our main interests is the metabolism of low molecular weight phenolic compounds and we have therefore tried to develop electrophoretic procedures for dealing with these materials and their degradation products. We find the method particularly useful for confirming structures which have already tentatively been deduced from chromatographic and spectrophotometric studies.[8–11]

The fundamentals of paper electrophoresis have already been outlined in the preceding chapter.

The potential gradients we use with phenols vary from 20 to 130 V/cm. These very high voltages are not essential but they do mean that separations can usually be effected within 15–45 min. The movement of a compound on an electrophoretogram is normally not recorded as an absolute mobility but relative to the mobility of a reference compound such as salicylic acid (M_{SA} value),[12] *p*-hydroxybenzoic acid[7] or DNP-glycine.[13] 5-Hydroxymethylfurfural can be used as a zero marker to compensate for electroendosmosis. This compound can readily be detected under u.v. light when it appears as a brown, absorbing spot. We normally detect phenols on the paper with u.v. light and/or by the use of diazotized *p*-nitroaniline spray reagent.[14]

It is obvious from the foregoing discussion on the electrophoretic mobility of phenols that the most important practical consideration is the choice of the electrolyte solution. These solutions can be conveniently divided into two main groups depending upon whether or not they contain components capable of forming charged complexes with phenols.

NON-COMPLEXING ELECTROLYTE SOLUTIONS

With these solutions the varying mobilities of phenols depend mainly on differences in the degrees of ionization of these molecules. These differences are usually very small, and in the case of compounds which possess only phenolic hydroxyl groups, not normally sufficient to allow resolution of mixtures. However, where there are substantial differences in the mass/charge ratios of such compounds separations can be effected. The non-complexing electrolytes can, of course, be used for separating a simple phenol from one possessing an additional ionized group (e.g. phenolic amine) and where there are relatively large differences in the dissociation constants of compounds within a particular group, such as the phenolic carboxylic acids, then such compounds can be separated from one another.

TABLE 2. M_{SA} VALUES FOR PHENOLS IN ACETATE, MOLYBDATE, PHOSPHATE, BORATE AND GLYCINE BUFFERS[12, 23]

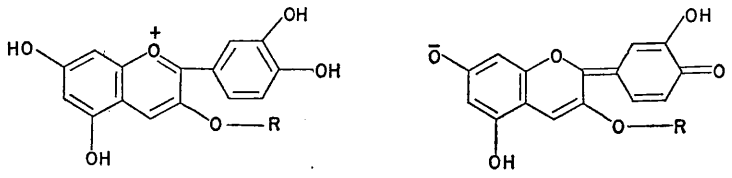
Compound	M_{SA} value				
	Acetate pH 5·2	Molybdate pH 5·2	Phosphate pH 7·2	Borate pH 10·0	Glycine pH 10·0
Quinol	0	0	0·01	*	*
Catechol	0	1·07	0·01	0·67	0·51
Resorcinol	0	0	0·01	0·35	0·44
Phloroglucinol	0	0	0·01	1·27	1·05
Pyrogallol	0	0·98*	0·01	0·75*	*
Hydroxyquinol	0	1·07	1·25 *	1·20*	1·16*
β-(3,4-Dihydroxyphenyl) L-alanine	0	0·81	0·01	0·72	*
o-Hydroxybenzoic acid	1·00	1·00	1·00	1·00	1·00
m-Hydroxybenzoic acid	0·81	0·79	0·85	1·05	0·95
p-Hydroxybenzoic acid	0·64	0·70	0·77	1·21	1·10
Protocatechuic acid	0·50	1·00	0·67	1·19	1·02
Gallic acid	0·45	1·03	0·59	1·20	0·93
p-Coumaric acid	0·34	0·44	0·58	0·98	0·86
Caffeic acid	0·20	0·79	0·50	1·04	1·05
Ferulic acid	0·21	0·21	0·42	0·87	0·77
Chlorogenic acid	0·33	1·03	0·44	0·83	0·74
p-Hydroxybenzaldehyde	0	0	0·27*	0·84*	0·79
Vanillin	0	0	0·27	0·79	0·76
Syringaldehyde	0	0	0·21	0·71	0·72
Arbutin	0	0	0	0·22	0·09
Phloridzin	0	0	0·08	0·42	0·14
Catechin	0	0·23	0	0·65	0·63
Quercetin	0	0·05	0	0·21	0
Myricetin	0	0	0	0·18	0
Luteolin	0	0	0	0·11	0
Kaempherol	0	0	0	0·12	0
Genistein	0	0	0·01	0·25	0·16
Irigenin	0	0	0·04	0·52	0·35
2-Naphthol	—	—	—	0·43	—
Coumarin	—	—	—	0	—
Umbelliferone	0	0	0·04	0·76	0·63
4-Methyl umbelliferone	0	0	0	0·52	0·50
Aesculetin	—	—	—	0·68	—
4-Methyl aesculetin	0	0·61	0·11	0·48	0·27
Aesculin	0	0	0·26	0·51	0·24

* Compound streaked.

Inspection of Table 2 shows that in *acetate buffer* (pH 5·2) simple phenols do not move (because they are not ionized at this pH) whereas phenolic carboxylic acids exhibit marked mobilities owing to the ionization of the carboxylic acid groupings. Phenolic compounds having other strongly acidic groups will also migrate in this buffer. For example, feeding resorcinol to broad bean seedlings produces *m*-hydroxyphenyl β-D-glucoside together with a compound which has been tentatively identified as *m*-hydroxyphenyl

β-D-glucoside-6′-sulphate.[15] These two compounds can be readily separated in the acetate buffer as the former is immobile at this pH whereas the latter has an M_{SA} value of 0·50.

Bayer[16, 17] observed that naturally occurring complexes of anthocyanins with aluminium and iron (e.g. protocyanin from cornflower) existed as anions and that they migrated in acetate buffer (pH 4·45) when a potential of 110V was applied across the paper. At this rather low voltage he was unable to demonstrate the electrophoretic migration of cyanin despite the positive charge carried by this molecule.[16] In 1960, however, Markakis,[18] using a higher voltage, showed that anthocyanins would move and that the direction of movement depended on the pH of the electrolyte. Thus antirrhinin (cyanidin-3-rhamoglucoside) had an isoelectric point of 6·0. On the acid side of this point the pigment moved towards the cathode and at pH values greater than 6·0, towards the anode. This can be explained by assuming that the oxonium form (I) is present in acid solution and tautomeric forms (e.g. (II)) in alkaline solution.



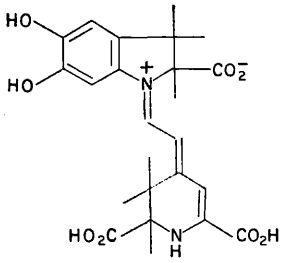
(I) (II)

(R = rhamoglucosyl)

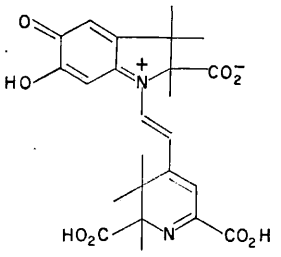
Schmidt and Schönleben[19] suggested that betanins might readily be recognized by their rapid electrophoretic migration towards the anode at pH 4·6. Lindstedt,[4] using *citrate buffers*, showed that the migration occurred within the pH range 2 to 7 thus confirming the presence of carboxyl groups in these pigments. The electrophoretic behaviour of the betanins was one of the first pieces of evidence which cast some doubt on the original suggestion that they were nitrogenous anthocyanin derivatives. Paper electrophoresis has certainly played an important role in the study of the distribution of betanins in the plant kingdom and the chemistry of these compounds.[1, 2] The procedure, using *aqueous formic acid* and *pyridine-formic acid electrolytes*, has been used widely by Dreiding and his collaborators who have recently shown that betanidin possesses one of the alternative structures (III or IV) given on page 116.[20]

Electrophoresis using a low pH electrolyte is also useful for detecting phenolic amino acids (e.g. DOPA) and phenolic amines (e.g. Dopamine) which occur in plant tissues. Such compounds exist in protonated forms in 0·1 M formic acid (pH 2·4) solution and hence move rapidly towards the cathode. Other phenolic compounds which may be present in plant tissue extracts are either immobile or move towards the anode at this pH.[21]

At neutrality or near neutrality, there is still little charge contribution from the phenolic hydroxyl groups unless ionization is facilitated by conjugation with electron-withdrawing groups. *Phosphate buffer* (pH 7·2) can be used with advantage for separating phenolic carboxylic acids from phenols as these two groups of compounds move a maximum distance apart at this pH.

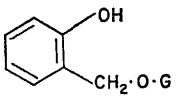


(III)

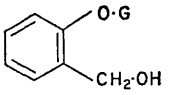


(IV)

Under alkaline conditions many polyphenols are readily oxidized and this is disadvantageous as it often results in tailing of the spots. Phenolic hydroxyl groups are usually strongly dissociated at pH values greater than 9 and, therefore, most simple phenolic compounds, in addition to phenolic carboxylic acids, will migrate fairly rapidly towards the cathode when, for example *glycine buffer* (pH 10·0) is used as an electrolyte. This buffer may be used to separate the two isomeric glucosides (V and VI) which are produced when saligenin is fed to broad bean seedlings.[9] Thus only (V) with the ionizable



(V)



(VI)

(G = glucosyl)

phenolic hydroxyl group can migrate (M_{SA} value 0·27) on a paper electrophoretogram at pH 10·0.

For a series of phenolic compounds with the same charge but with increasing molecular weight there is generally a decrease in electrophoretic mobility under alkaline conditions in the absence of complexing agents. For example, with the phenolic aldehydes (Table 2) the relative mobilities are *p*-hydroxybenzaldehyde > vanillin > syringaldehyde in glycine buffer. Conversely, for molecules of similar molecular weight increase in mobility roughly parallels increase in charge. Thus trihydroxybenzene derivatives normally move more rapidly than dihydroxy derivatives. These basic rules are, however, often quite inadequate to explain the electrophoretic behaviour of many compounds due to complications arising from group interaction. Thus the order of movement of the isomeric monohydroxybenzoic acids in glycine

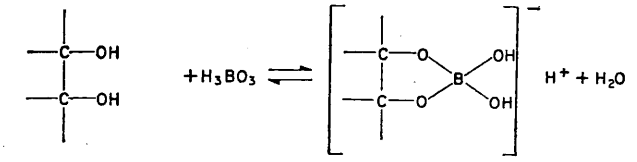
buffer (pH 10·0) is *para* > *ortho* > *meta*. This is difficult to explain and is not the same as in acetate buffer (pH 5·2).

The electrophoresis of coumarin derivatives using a *veronal–sodium acetate buffer* (pH 8·6) has been studied by Krebs and Wankmüller.[22] Despite the low voltages used by these workers the coumarin derivatives moved quite rapidly. Similar results (cf. M_{SA} values for β-naphthol and umbelliferone) have been obtained by Pridham[12, 23] using *borate buffer* (pH 10·0) (Table 2). (*N.B.* With the exception of aesculetin, complex formation is not possible with the coumarin derivatives listed in Table 2.) These high mobilities may be due to the increased acidities of the 7-hydroxyl groups resulting from conjugation with the carbonyl groups in the lactone rings.

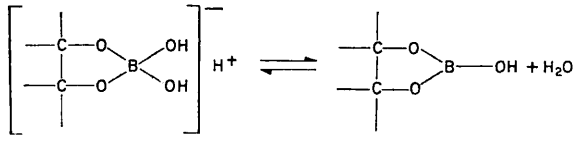
COMPLEXING ELECTROLYTE SOLUTIONS

It has already been mentioned that the ionic character of some phenols may be increased by complex formation. Thus phenols possessing *o*-dihydroxyl groups will form charged complexes with a number of anions which results in a general increase in the electrophoretic mobilities of these compounds and, in particular, a much better separation of *o*-dihydric phenols from those with other hydroxyl arrangements.

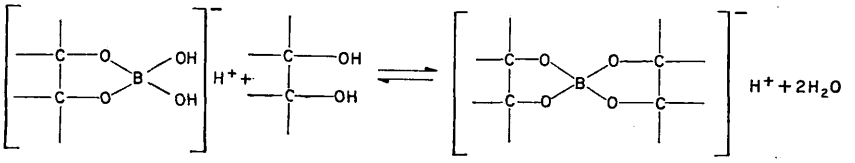
Böeseken[24] originally showed that catechol and substituted catechols increased the conductivity (acidity) of boric acid solutions but not *m*- or *p*-dihydroxybenzenes or their derivatives. Hermans[25] and Vermaas[26] concluded that boric acid reacted with suitable diols as follows:



(VII)



(VIII)



(IX)

The structure of the borate ion is now known to be $[B(OH)_4]^-$[27, 28].

The 1 : 1 complex (VII) which is readily formed is a relatively weak acid which can lose water to form a neutral compound (VIII). However, if the hydroxyl arrangement of the diol is favourable a strongly acidic 2 : 1 complex (IX) can be produced. Such a complex of catechol with boric acid has been isolated by Böeseken as a potassium salt. Isbell and his collaborators[29] have stated that in the case of borate complexes formed with carbohydrates that type (IX) should be formed in the presence of excess carbohydrate and excess borate should favour type (VII); the same rules presumably apply to phenols. However, a number of difficulties do arise when considering complex formation in relation to the paper electrophoresis of phenols and these will be discussed later.

The ability of *o*-dihydric phenols to form negatively charged complexes was first exploited by Michl[30] who described the high voltage paper electrophoresis of catechin, methyl gallate, protocatechualdehyde, pyrogallol and a tannin extract using an *alkaline–borate* solution. This was shortly followed by an account of the electrophoretic migration of flavonoids by Hashimoto *et al.*[31] using a solution of sodium borate. The latter workers tried to show that the rate of movement was directly related to the total number of *o*-dihydroxyl groups in the aglycones and *cis*-hydroxyl groups in the glycosidic moieties. In their table of mobilities, however, they appear in some cases to have miscalculated the numbers of these groups and the relationship is therefore not conclusive.

The electrophoresis of phenols using alkaline borate solutions has also been studied by Coulson and Evans[13] who were mainly interested in phenolic carboxylic acids (Table 3) and Pridham[12, 23] who examined members from several different groups of phenols (Table 2).

Boric acid solutions (pH 4–5) may also be used as electrolytes, but the phenol–borate complexes do not migrate as rapidly as with borate solutions of higher pH (Table 4).[23]

The formation of 1 : 1 and 2 : 1 phenol–borate complexes have already been mentioned. The nature of the complex formed on a paper electrophoretogram is, however, difficult to establish. The phenol/borate ratio in a spot on an electrophoretogram is relatively high and this, according to Isbell *et al.*,[29] should favour the 2 : 1 complex. If this is the case then one might also expect mixed complexes to be formed between two different phenols and borate. In such a case the electrophoresis of a mixture of two *o*-dihydric phenols, $R'(OH)_2$ and $R''(OH)_2$, should produce three spots due to the formation of:

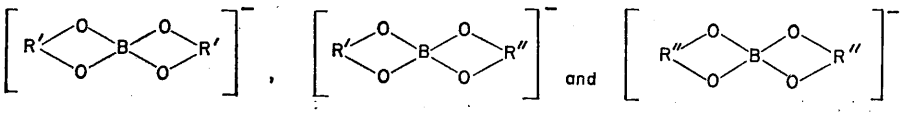


TABLE 3. THE MOBILITIES OF PHENOLS RELATIVE TO 2,4-DINITROPHENYL GLYCINE IN BORATE BUFFER[18]

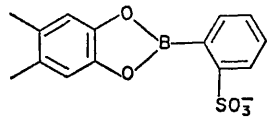
	Relative mobility
Homocatechol	1·02
Saligenin	1·02
3,4-Dihydroxy-ω-chloroacetophenone	0·70
o-Hydroxybenzoic acid	1·28
o-Hydroxyphenylpropionic acid	1·12
m-Hydroxyphenylpropionic acid	1·05
p-Hydroxyphenylpropionic acid	1·08
p-Hydroxyphenylpyruvic acid	1·13
o-Hydroxy-*cis*-cinnamic acid	1·08
o-Hydroxy-*trans*-cinnamic acid	1·20
m-Hydroxycinnamic acid	1·15
o-Hydroxyphenyl-glyoxylic acid	1·35
2,3-Dihydroxybenzoic acid	1·46
2,4-Dihydroxybenzoic acid	1·60
2,5-Dihydroxybenzoic acid	1·20
2,6-Dihydroxybenzoic acid	1·37
3,4-Dihydroxybenzoic acid	1·70
3,5-Dihydroxybenzoic acid	1·34
2,3-Dihydroxyphenylacetic acid	1·90
2,5-Dihydroxyphenylacetic acid	1·1–1·55 (streak)
3,4-Dihydroxyphenylacetic acid	1·70
2,3-Dihydroxyphenylpropionic acid	1·60
2,5-Dihydroxyphenylpropionic acid	1·25–1·80 (streak)
3,4-Dihydroxyphenylpropionic acid	1·55
2,5-Dihydroxycinnamic acid	1·3–1·9 (streak)
3,4-Dihydroxycinnamic acid	1·49
2,5-Dihydroxyphenylpyruvic acid	0·88
2,5-Dihydroxyphenylacetic acid lactone	0·9–1·88 (streak)

TABLE. 4. M_{SA} VALUES FOR PHENOLS IN AQUEOUS BORIC ACID SOLUTION (pH 4·4) (CF. TABLE 2)[23]

	M_{SA} value
Resorcinol	0·07
Catechol	0·37
Ferulic acid	0·40
Caffeic acid	0·51
Catechin	0·25

Fortunately, however, there is no evidence of this phenomenon on paper electrophoretograms. If it did occur then the use of borate for the fractionation of phenol mixtures would be fraught with difficulties.

Garegg and Lindberg[32] have described the separation of carbohydrates using electrolytes containing *sulphonated phenylboronic acid.* We[33] have also used this reagent in the hope that the complexes (X) formed would bring about a large increase in the mobilities of the higher molecular weight phenolic compounds owing to the presence of the strongly dissociated sulphonic acid grouping.



(X)

Figure 1 shows the relative rates of movement of a number of phenolic compounds in this electrolyte at various pH values but there appears to be no great advantage in using this system in preference to borate.

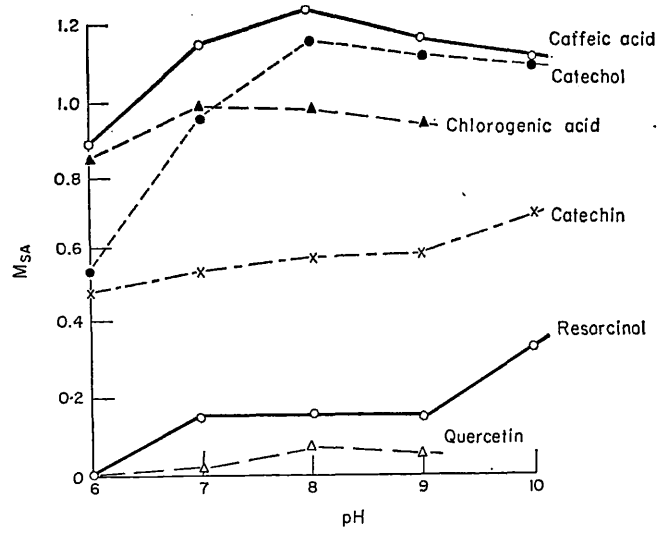


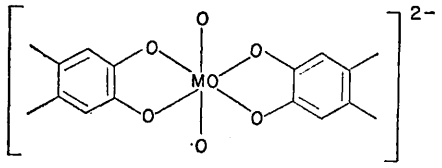
FIG. 1 M_{SA} values of phenols in sulphonated phenylboronic acid solutions.

Molybdate ions react quite specifically with *o*-dihydric phenols to form brown, negatively charged complexes. Hence *ammonium* or *sodium molybdate* solutions (pH 5·2) may be used for the electrophoresis of these compounds (Table 2).[12] At this pH only phenolic carboxylic acids and *o*-dihydric phenols will migrate (and phenols with "activated" hydroxyl groups). With *o*-dihydric phenols use of this electrolyte has the advantage that the spots

TABLE 5. THE MOBILITIES OF PHENOLS RELATIVE TO *p*-HYDROXYBENZOIC ACID IN PHOSPHATE BUFFER CONTAINING COMPLEXING INORGANIC ANIONS[7]

pH	Complexing ion	Relative Mobility					
		Catechol	Pyrogallol	3,4-Dihydroxybenzaldehyde	3,4-Dihydroxybenzoic acid	3,4,5-Trihydroxybenzoic acid	*o*-Hydroxybenzoic acid
7	None	0·04	0·04	0·29	0·90	0·78	1·23
8	None	0·03	0·06	0·59	0·89	0·75	1·31
7	Molybdate	1·15	1·02	1·13	1·57	1·42	1·18
7	Tungstate	1·14	1·22	1·05	1·65	1·53	1·21
7	Phosphomolybdate	1·08	0·62	1·12	1·62	1·44	1·16
7	Phosphotungstate	1·11	1·06	1·11	1·88	1·63	1·28
7	Arsenite	0·07	0·07	0·47	0·93	0·77	1·06
8	Arsenite	0·11	0·04	0·60	1·06	0·94	1·28
7	Vanadate	0·98	1·38	1·08	1·38	1·68	1·22
8	Vanadate	1·25	1·22	0·77	1·77	1·68	1·16
7	Stannate	0·76	0·73	0·77	1·36	1·27	1·20

are coloured and progress down the paper can therefore be observed. Halmekoski[7] has carried out a spectrophotometric investigation of phenol–molybdate complexes in solution and the main complex appears to have the structure (XI). As with borate, however, no evidence is available to suggest



(XI)

that mixed complex formation can occur on paper electrophoretograms. Hence there is some doubt as to whether Halmekoski's results offer a complete explanation of complex formation under the conditions found on a paper electrophoretogram.

Halmekoski[7] has also carried out electrophoresis using *sodium molybdate in phosphate buffer* (pH 7) and shown that *phosphomolybdate*, *tungstate*, *phosphotungstate*, *vanadate* and *stannate solutions* (all in phosphate buffer at pH 7 or 8) also form fast moving complexes with *o*-dihydric phenols. *Arsenite* showed only a slight tendency to complex (Table 5). Spectrophotometric studies again showed that the main complexes formed in solution with tungstate, vanadate and stannate were of the 2 : 1 type. Tungstate and 3,4,5-trihydroxybenzoic acid, however, appeared to form mainly a 1 : 1 complex. The situation with vanadate is complicated as in addition to 2 : 1 complexes 5 : 1 can also probably be formed with catechol, pyrogallol and probably other phenols. The phenol when in excess may also reduce the vanadate ion to vanadyl (VO_2^{2+}) which in turn can also form complexes.

TABLE 6. M_{SA} VALUES OF PHENOLS IN BASIC COPPER ACETATE SOLUTION pH 5·3 (MIGRATION TOWARDS ANODE)[33]

	M_{SA} value
2,6-Dimethoxyphenol	0
Catechol	0·05
Monomethyl quinol	0·26
Saligenin	0·30
Phloroglucinol	0·30
Resorcinol	0·31

In this laboratory the electrophoretic migration of some phenols using *basic copper acetate* (pH 5·3) as an electrolyte has been investigated (Table 6).[33] In this solution the compounds moved towards the anode in direct contrast to carbohydrates which form cationic complexes with the copper reagent.[34] This behaviour of phenols, at present, cannot be explained.

Theander,[35] who was interested in the separation of lignin degradation products, has described a method for the separation of phenolic aldehydes using a *bisulphite buffer* at pH 4·7. Migration is dependent on the formation of ionized hydroxysulphonic acid derivatives (Table 7).

In an endeavour to speed up the migration of the larger phenols these compounds have been coupled with *diazotized sulphanilic* and *p-aminobenzoic acids*. The resulting acidic azo dyes were then subjected to electrophoresis using a borate (pH 10) buffer[36] (cf. Franc and Wurst[37]). Table 8

TABLE 7. MOBILITIES OF PHENOLIC ALDEHYDES RELATIVE TO VANILLIN IN BISULPHITE BUFFER pH 4·7[35]

	M_V value
2,4-Dihydroxybenzaldehyde	0·82
4-Hydroxy-3,5-dimethoxybenzaldehyde	0·95
2-Hydroxybenzaldehyde	0·95
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	1·00
3,4-Dihydroxybenzaldehyde	1·00
2-Hydroxy-3-methoxybenzaldehyde	1·10
3-Hydroxybenzaldehyde	1·16
3-Aldehydo-4-hydroxy-5-methoxybenzaldehyde	1·53

shows the M_{SA} values obtained with some phenols and the corresponding benzene-azo-*p*-carboxylates. It is apparent that introduction of the carboxyl group greatly enhances mobility, but, as might be expected, offers little benefit with regard to the fractionation of mixtures of these phenols. The method might, however, be useful in conjunction with paper chromatography.

TABLE 8. COMPARISON OF M_{SA} VALUES OF PHENOLS AND THEIR BENZENE-AZO-*p*-CARBOXYLATE DERIVATIVES IN BORATE BUFFER (pH 10)[36]

	Phenol	Derivative
Arbutin	0·22	0·50
Phloridzin	0·42	0·61
Syringin	0·09	0·34
Kaempferol	0·12	0·23
Quercetin	0·15	0·32

A preliminary identification of a phenol could be achieved by chromatographic comparison with an authentic standard, both compounds being located on the paper with an acidic diazonium reagent. The coloured spots of azo dye could then be eluted from the paper and again compared on an electrophoretogram.

With regard to future development, most of the practical aspects of this topic have probably been fully exploited. The future discovery of reagents which will form charged complexes with groups other than *o*-dihydroxyl is, however, a possible line of advancement.

REFERENCES

1. H. REZNIK, *Planta*, **49**, 406 (1957).
2. A. S. DREIDING in *Recent Developments in the Chemistry of Natural Phenolic Compounds*, p. 194, ed. W. D. Ollis, Pergamon Press, Oxford (1961).
3. J. HALMEKOSKI, *Suomen. Kem.* **35B**, 115 (1962).
4. G. LINDSTEDT, *Acta Chem. Scand.* **10**, 698 (1956).
5. S. P. SPRAGG in *Phenolics in Plants in Health and Disease*, p. 17, ed. J. B. Pridham, Pergamon Press, London (1960).
6. J. FRANC and M. WURST, *Coll. Czech. Chem. Comm.* **24**, 857 (1959).
7. J. HALMEKOSKI, *Ann. Acad. Scient. Fennicae*, A, II, 96 (1959).
8. J. B. PRIDHAM, *Chem. and Ind.* 1172 (1962).
9. J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **87**, 218 (1963).
10. NORMA J. MACLEOD and J. B. PRIDHAM, *Biochem. J.* **88**, 45P (1963).
11. E. J. BOURNE, NORMA J. MACLEOD and J. B. PRIDHAM, *Phytochem.* **2**, 225 (1963).
12. J. B. PRIDHAM, *J. Chromatog.* **2**, 605 (1959).
13. C. B. COULSON and W. C. EVANS, *J. Chromatog.* **1**, 374 (1958).
14. T. SWAIN, *Biochem. J.* **53**, 200 (1953).
15. J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **82**, 44P (1962).
16. E. BAYER, *Chem. Ber.* **91**, 1115, (1958).
17. E. BAYER, *Chem. Ber.* **92**, 1062 (1959).
18. P. MARKAKIS, *Nature, Lond.* **187**, 1092 (1960).
19. O. T. SCHMIDT and W. SCHÖNLEBEN, *Naturwiss.* **43**, 159 (1956).
20. A. S. DREIDING, *Helv. Chem. Acta*, **45**, 640 (1962).
21. R. S. ANDREWS and J. B. PRIDHAM, unpublished results.
22. K. KREBS and A. WANKMÜLLER, *Naturwiss.* **40**, 623 (1953).
23. J. B. PRIDHAM, unpublished results.
24. J. BÖESEKEN, *Adv. Carbohydrate Chem.* **4**, 189 (1949).
25. P. H. HERMANS, *Z. anorg. Chem.* **142**, 83 (1925).
26. N. VERMAAS, *Rec. Trav. Chim.* **51**, 67 (1932).
27. J. O. EDWARDS, G. C. MORRISON, V. F. ROSS and J. W. SCHULTZ, *J. Amer. Chem. Soc.* **77**, 226 (1955).
28. M. FORNASERI, *Period. Miner.* **18**, 103 (1949). **19**, 157 (1950).
29. H. S. ISBELL, J. F. BREWSTER, N. B. HOLT and H. L. FRUSH, *J. Res. Nat. Bur. Stand.* **40**, 129 (1948).
30. H. MICHL, *Monatsh.* **83**, 737 (1952).
31. Y. HASHIMOTO, I. MORI and M. KIMURA, *Nature, Lond.* **170**, 975 (1952).
32. P. J. GAREGG and B. LINDBERG, *Acta Chem. Scand.* **15**, 1913 (1961).
33. J. B. PRIDHAM and F. C. SAWYER, unpublished results.
34. F. SEARLE and H. WEIGEL, unpublished results.
35. O. THEANDER, *Acta Chem. Scand.* **11**, 717 (1957).
36. NORMA J. MACLEOD and J. B. PRIDHAM, unpublished results.
37. J. FRANC and M. WURST, *Coll. Czech. Chem. Comm.* **25**, 663 (1960).

B16

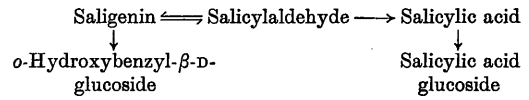
[*Reprinted from the Proceedings of the Biochemical Society*, 14–15 *May* 1964.
Biochem. J., 1964, Vol. 92, No. 1, 20–21 P.]

Oxidation and Reduction of Aromatic Compounds by Plants

By J. B. PRIDHAM and MARGARET YOUNG. (*Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey*)

Pridham & Saltmarsh (1963) observed that plant tissues rapidly converted saligenin (*o*-hydroxybenzyl alcohol) to *o*-hydroxybenzyl-β-D-glucoside but little of the naturally occurring isomer, salicin (*o*-hydroxymethylphenyl-β-D-glucoside), was produced. This suggested that saligenin was not the direct precursor of salicin in plants and the possibility that this compound was formed by the reduction of an *O*-glucosyl derivative of salicylaldehyde was considered. Salicylaldehyde occurs naturally in plants (Karrer, 1958) and it has been isolated as a glucoside, helicin, from *Spiraea kamtschatica* (Beijerinck, 1899). According to Miwa, Nakamura & Shibata (1957) salicylaldehyde-β-D-glucoside is formed when leaf disks from various plant species are infused with the aldehyde.

The metabolism of saligenin, salicylaldehyde and salicylic acid in relation to salicin biosynthesis is now being studied. Feeding experiments using *Vicia faba* seedlings and the three aglycones have revealed that the following pathways are operative:



Klämbt (1962) has shown that benzoic acid can also be converted to salicylic acid and the corresponding β-D-glucoside by *Helianthus* hypocotyls.

The occurrence and metabolism of monocyclic aromatic compounds in higher plants will be reviewed.

Beijerinck, M. W. (1899). *Zbl. Bakt. P.* (II), **5**, 425; (1899) *Chem. Zentr.* II, **70**, 259.

Karrer, W. (1958). *Konstitutionen und Vorkommen der organischen Pflanzenstoffe*, p. 157. Basel: Birkhäuser Verlag.

Klämbt, H. D. (1962). *Nature, Lond.*, **196**, 491.

Miwa, T., Nakamura, S. & Shibata, A. (1957). *Kôso Kagaku Shinpojiuma*, **12**, 48; (1958). *Chem. Abstr.* **52**, 1314.

Pridham, J. B. & Saltmarsh, M. J. (1963). *Biochem. J.* **87**, 218.

REPRINTED FROM
ADVANCES IN CARBOHYDRATE CHEM., VOL. 20
© 1965
ACADEMIC PRESS INC., NEW YORK

PHENOL–CARBOHYDRATE DERIVATIVES IN HIGHER PLANTS

By J. B. Pridham

Department of Chemistry, Royal Holloway College,
University of London, Englefield Green, Surrey, England

I. Introduction

One of the most abundant and diverse groups of naturally occurring, organic materials is that of the phenolic compounds present in the tissues of higher plants. These compounds range from polymers of high molecular weight, such as lignin, to relatively simple, benzene derivatives. Lignin is an insoluble, structural component of woody tissues; but, in addition, a complex mixture of water-soluble phenolic compounds is found within the cells of most plant organs, and the great majority are chemically bonded to carbohydrates. There is a great deal of evidence to suggest that lignin is also bonded to carbohydrate in the plant cell-wall.[1]

The purpose of this article is to bring together the known chemical and biochemical facts regarding these compounds, and to treat them, not as phenolic derivatives, but as carbohydrate derivatives. Such an approach is unusual, but the carbohydrate moieties of these molecules have, in the past, been largely ignored by chemists more intent on investigating the exciting, new aromatic and heterocyclic structures being discovered in

(1) T. Poletz, *Sitzber. Heidelberg. Akad. Wiss. Math. Naturw. Kl. Abhandl.*, **10**, (1940); *Chem. Abstracts*, **42**, 8463 (1948); C. M. Stewart, *Tappi*, **40**, 244 (1957); R. E. Kremers, *Ann. Rev. Plant Physiol.*, **10**, 185 (1959); H. Brownell and K. L. West, *Pulp Paper Mag. Can.*, **62**, T374 (1961).

the plant kingdom. The impression gained on reading some of the early work is that the combined sugar was regarded only as something to be removed and discarded at the earliest possible stage, before work was commenced on the phenol itself. Often, the sugar was entirely ignored or identified inadequately; it was, of course, extremely difficult to separate and identify mixtures of monosaccharides before the advent of partition chromatography. Some of the methods of isolation were open to criticism, including the use of aqueous extraction of plant material at high temperatures and the failure to inactivate the tissue hydrolases properly; both of these may bring about the degradation of carbohydrate derivatives.

At the present time, a great deal more attention is being paid to these structures, and all of the modern methods are being applied to their elucidation. It is still, however, often difficult in many cases to perform a rigorous characterization, because of limited amounts of starting material. For this reason, the absolute configurations of combined monosaccharide residues are rarely ascertained, and ring sizes and anomeric configurations are not usually known with certainty. In some of the early literature, there are obvious errors which need correction, particularly in articles published before the general introduction of chromatography. There is also some confusion in the literature because a number of different systems of nomenclature have been used to describe these compounds.

A complete catalog of the many, naturally occurring, phenol–carbohydrate derivatives is not intended and, indeed, would be out of the question in an article of this type. Comprehensive lists of these compounds, and a discussion of their chemistry, may be found in publications by McIlroy,[2] Karrer,[3] Stoll and Jucker,[4] Geissman,[5,6] and Dean.[7] An attempt will be made, however, to describe the principal carbohydrates found in combination with phenols, and to draw attention to the structural diversity within this group of compounds and the interesting biochemistry involved. In addition, the special methods used for the elucidation of the structures of combined carbohydrates will be discussed.

(2) R. J. McIlroy, "The Plant Glycosides," Edward Arnold & Co., London, 1951.
(3) W. Karrer, "Konstitution und Vorkommen der organischen Pflanzenstoffe," Birkhauser Verlag, Basel, 1958.
(4) A. Stoll and E. Jucker, in "Encyclopedia of Plant Physiology," W. Ruhland, ed., Springer-Verlag, Berlin, 1958.
(5) T. A. Geissman, in "The Chemistry of Flavonoid Compounds," T. A. Geissman, ed., Pergamon Press, Oxford, 1962.
(6) T. A. Geissman, in "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, eds., Elsevier Publishing Co., Amsterdam, 1963, Vol. 9, p. 211.
(7) F. M. Dean, "Naturally Occurring Oxygen Ring Compounds," Butterworths, London, 1963.

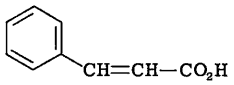
II. Nature of the Phenol–Carbohydrate Derivatives

1. *Types of Linkage*

In higher plants, the mono- and oligosaccharide residues found in combination with phenolic compounds may be divided into two main groups: (1) the (more numerous) glycosides, and (2) the phenolic carboxylic esters of sugars, esterification normally involving the hydroxyl group on the anomeric carbon atom. Group 1 consists largely of phenolic glycosides, but there are also examples of phenolic alcohols where the alcoholic group is glycosylated. In group 2, there are, reportedly, compounds wherein a secondary alcoholic group of a carbohydrate is esterified with a phenolic carboxylic acid. Ester and glycosidic linkages can both occur in the same compound. Examples of these various types of derivative will be given below. The *C*-glycosyl compounds[8] will not be discussed in the present article.

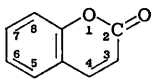
2. *The Combined Phenols*

The more complex groups of phenols found in combination with carbohydrates in plants are summarized in formulas (1) to (13). In addition, there are also simple mono-, di-, and tri-hydric phenols existing as glycosides; some of these phenols possess additional functional groups, such as methoxyl, primary hydroxyl, and aldehyde groups. Hydroxybenzoic acids and some of their aliphatic esters, and hydroxycinnamic acids, are found as glycosides, and the acids themselves may also be esterified with mono- or oligo-saccharides.



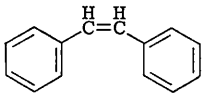
Cinnamic acids

(1)



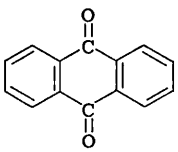
Coumarins

(2)



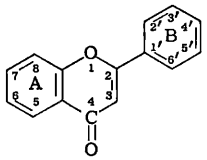
Stilbenes

(3)



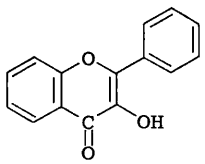
Anthraquinones

(4)



Flavones

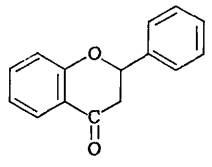
(5)



Flavonols

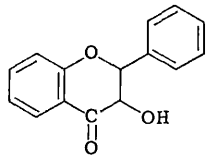
(6)

(8) L. Hörhammer and H. Wagner, in "Recent Developments in the Chemistry of Natural Phenolic Compounds," W. D. Ollis, ed., Pergamon Press, Oxford, 1961, p. 185. L. J. Haynes, *Advan. Carbohydrate Chem.*, **18**, 227 (1963); **20**, 357 (1965).



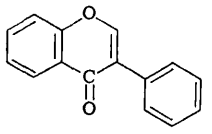
Flavanones

(7)



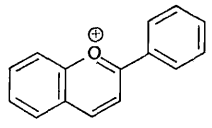
Flavanonols

(8)



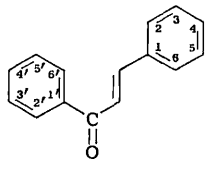
Isoflavones

(9)



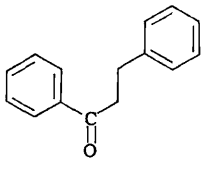
Anthocyanidins
(Flavylium ion)

(10)



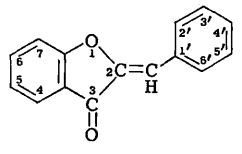
Chalcones

(11)



Dihydrochalcones

(12)



Aurones
(Benzalcoumaranones)

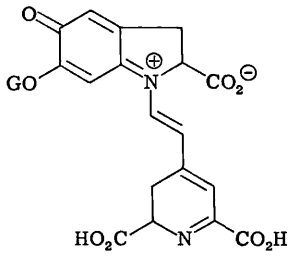
(13)

With the flavonoid derivatives (**5**) to (**13**), a great diversity of structures is possible. These C_{15} compounds commonly have a number of hydroxyl groups on both the A and the B rings, and the flavonols (**6**), flavanonols (**8**), and the majority of anthocyanidins (**10**) also have a hydroxyl group at C-3. This polyhydric character, together with the fact that a number of different monosaccharides may form glycosides, either as single residues or as oligosaccharides, means that many glycosidic forms are possible; these do, indeed, exist in plant cells. Further complications arise when, in some anthocyanidin and flavonol glycosides, the glycon is also acylated with a phenolic carboxylic acid. There does, however, appear to be a general pattern with regard to substitution on flavonoid hydroxyl groups. Thus, with the anthocyanins,[9] the hydroxyl group at C-3 is always glycosylated, and the second hydroxyl group to be substituted is usually at C-5. Glycosylation of other hydroxyl groups is unusual. The flavonols (**6**)

(9) That is, glycosides of anthocyanidin (**10**).

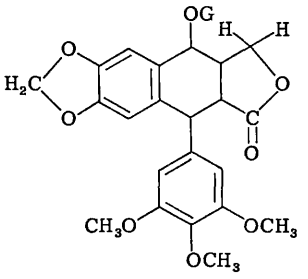
constitute the largest and most variable class of flavonoid glycosides. Here again, the hydroxyl group at C-3 is usually, but not always, glycosylated. The hydroxyl group at C-7 is, normally, the secondary position for glycoside formation, and C-3′ and C-4′ may also carry glycosyloxy groups. Of the relatively few flavanonol (**8**) glycosides known, the majority are 3-glycosides. The flavones (**5**) and flavanones (**7**) have no hydroxyl group at C-3, and glycosylation occurs mainly at the hydroxyl group at C-7 and, occasionally, at that at C-5. Substitution at the 4′-hydroxyl group is also not uncommon with the flavones. The isoflavones (**9**) are mainly glycosylated at the hydroxyl group on C-7, and some 4′-glycosides have also been isolated. Chalcones (**11**) and dihydroxychalcones (**12**) are structurally related to flavones and flavanones, respectively, and, as would be expected, glycosylation occurs mainly at the equivalent hydroxyl group at C-4′ of (**11**) and (**12**). In the aurones (**13**), C-6 corresponds to C-7 in the flavones, and substitution is found mainly at the hydroxyl group at C-6.

Other phenolic compounds found naturally as glycosides, but about which less is known, include betanidin, a red, nitrogenous pigment belonging to a class of compounds known as the betacyanins, found in the Centrospermae. These pigments have been reviewed by Dreiding,[10] and betanidin D-glucoside (betanin) has been shown to have structure (**14**) or a tautomeric form thereof.[11] In addition, a few naturally occurring lignan glycosides have been reported to occur in plants [two such examples are (**15**) and (**16**)],[12a] and a 3-D-glucosyl derivative of epicatechin (**17**) has also been described.[12b]



Betanin

(14)



Picropodophyllin D-glucoside

(15)

(10) A. S. Dreiding, Ref. 8, 1962, p. 194.

(11) T. J. Mabry, H. Wyler, G. Sassu, M. Mercier, J. Parikh, and A. S. Dreiding, *Helv. Chim. Acta*, **45**, 640 (1962). See, however, *This Vol.*, p. 480.

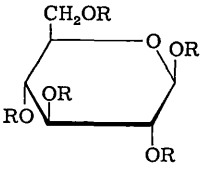
(12a) W. N. Hearon and W. S. MacGregor, *Chem. Rev.*, **55**, 957 (1955).

(12b) A. M. Gakhokidze, *Zh. Prikl. Khim.*, **19**, 1197 (1946); *Chem. Abstracts*, **42**, 559 (1948).

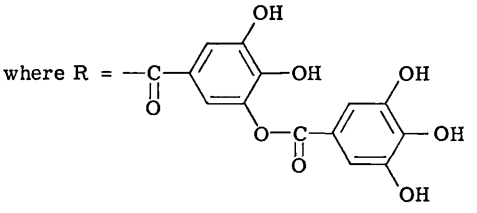
pyranosyl gallate, was obtained from Chinese rhubarb by Gilson,[28] and the structure of the ester was later determined by degradation and synthesis.[29] Initial observations suggest that glycosides of hydroxybenzoic acids may be quite common in plant tissues.[30]

While considering glycosides of phenolic acids, it should be noted that the phenolic amino acid, β-(3,4-dihydroxyphenyl)-L-alanine, which occurs free in high concentrations in *Vicia faba* (broad bean), is also present as the 3-*O*-β-D-glucopyranosyl derivative in the seed testas of this plant.[31] It would be interesting to know whether L-tyrosine also exists combined with D-glucose.

Galloyl esters of D-glucose are related to the gallotannins, members of the group of hydrolyzable tannins which are of widespread occurrence in the plant kingdom. The early work by Fischer, using hydrolytic and methylation procedures, suggested that Chinese gallotannin (obtained from *Rhus semialata* galls) might be a mixture of poly-*O*-galloylated D-glucose derivatives, and it was noted that the average composition corresponded to that of a penta-*O*-*m*-digalloyl-β-D-glucose (**19**).[32] In 1956,



(19)



(28) E. Gilson, *Compt. Rend.*, **136**, 385 (1903).
(29) E. Fischer and M. Bergmann, *Ber.*, **51**, 1760 (1918).
(30) R. K. Ibrahim, G. H. N. Towers, and R. D. Gibbs, *J. Linnean Soc. London Botany*, **58**, 223 (1962).
(31) T. Nagasawa, H. Takagi, K. Kawakami, T. Suzuki, and Y. Sahashi, *Agr. Biol. Chem.* (Tokyo), **25**, 441 (1961); *Chem. Abstracts*, **55**, 24942 (1961). R. S. Andrews and J. B. Pridham, *Nature*, **205**, 1213 (1965).
(32) E. Fischer, *Ber.*, **46**, 3253 (1913); **52**, 809 (1919).

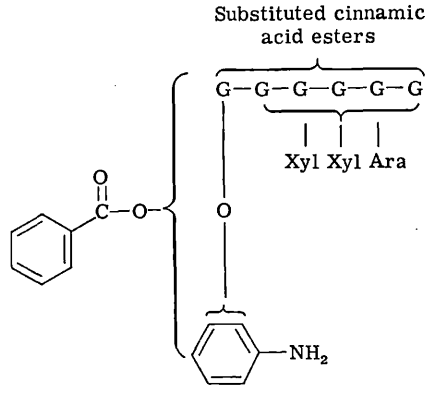
White,[33] on the basis of hydrolysis data, stated that Chinese gallotannin might well contain a tri- or tetra-saccharide core, instead of just D-glucose. In the same year, Grassmann and coworkers[34] concluded, again from the results of hydrolysis studies, that Staghorn sumac (*Rhus typhina*) tannin contains a poly-*O*-galloyl tetrasaccharide, one molecule of the latter consisting of two residues of D-glucose and one each of L-arabinose and L-rhamnose. In an effort to clear up the disagreement regarding the nature of the carbohydrate core in these tannins, R. D. Haworth and coworkers prepared, from *Aspergillus niger*, a galloyl esterase (tannase) which was virtually free of carbohydrase activity.[35] Using this enzyme, and a methanolysis procedure for cleaving depside linkages,[36] Chinese, Staghorn sumac, and Sicilian sumac (*Rhus coriaria*) tannins were degraded and shown to contain only a D-glucose core. All of these tannins appear to be mixtures of closely related substances, but the main component in each contains a penta-*O*-galloyl-β-D-glucose nucleus to which three or four additional galloyl groups are attached by depside linkages.[37] These observations showed that the original findings of Fischer[31] were essentially correct.

Another group of phenolic acids which, combined with D-glucose, appears to be widespread in plant tissues is that of derivatives of cinnamic acid (1). Until quite recently, it was believed that the quinic acid esters of these compounds, for example chlorogenic acid (3-caffeoylquinic acid), were the most common, but D-glucose derivatives are now frequently reported. Harborne and Corner[38] believed that many of the reputed quinic acid esters, detected by chromatographic surveys in the past, may well be D-glucose esters. The cinnamic acids most frequently encountered are *p*-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid). The first three are found in many plants as the corresponding β-D-glucopyranosyl cinnamates.[38] The structure of the D-glucose moieties in these compounds is based only on hydrolysis by almond emulsin and the oxidation of the liberated sugar by the specific D-glucose oxidase (β-D-glucose: O_2 oxidoreductase). There is, however, no reason to suppose that the structure assigned is incorrect. The sinapoyl ester has, so far, been detected only in *Brassica*

(33) T. White, in "The Chemistry of the Vegetable Tannins," Soc. of Leather Trades' Chemists, Croydon, England, 1956, p. 13.
(34) W. Grassmann, G. Stiefenhofer, and H. Endres, *Chem. Ber.*, **89**, 454 (1956).
(35) E. Haslam, R. D. Haworth, K. Jones, and H. J. Rogers, *J. Chem. Soc.*, 1829 (1961).
(36) E. Haslam, R. D. Haworth, S. D. Mills, H. J. Rogers, R. Armitage, and T. Searle, *J. Chem. Soc.*, 1836 (1961).
(37) R. Armitage, G. S. Bayliss, J. W. Gramshaw, E. Haslam, R. D. Haworth, K. Jones, H. J. Rogers, and T. Searle, *J. Chem. Soc.*, 1842 (1961).
(38) J. B. Harborne and J. J. Corner, *Biochem. J.*, **81**, 242 (1961).

oleracea leaves.[38] A D-glucose ester of 4-methoxycinnamic acid may be present in *Polygala senega*.[39]

Aryl D-glucosides in the cinnamic series appear to be much less common than the D-glucose esters, and have only been detected in a few plant species. Melilotoside, for example, was discovered in *Melilotus* spp. by Charaux.[40] By synthesis, Shinoda and Imaida[41] confirmed the structure of this compound as being 2-(β-D-glucopyranosyloxy)-*trans*-cinnamic acid. The *cis* isomer, which is *o*-coumarinyl β-D-glucoside, has been found in clover by Kosuge.[42] Flaxseed hulls contain a complex phenolic polymer which, on alkaline methanolysis, yields the methyl esters of 4-(β-D-glucopyranosyloxy)cinnamic acid (namely, linocinnamarin)[43] and 4-(β-D-glucopyranosyloxy)-3-hydroxycinnamic acid (namely, linocaffein).[44] 3-Hydroxy-3-methylglutaric acid, unknown D-glucosides, and phenolic derivatives are also released from the polymer by treatment with alkali. It is believed that the identified components of the polymer are joined, by ester linkages, to a complex macromolecule. In this connection, it is appropriate to mention that Kodicek and Wilson[45] have described the isolation of a material of high molecular weight (niacytin) from wheat bran; to it, they have assigned the tentative structure (**20**):



where G = D-glucosyl.

(20)

(39) J. J. Corner, J. B. Harborne, S. G. Humphries, and W. D. Ollis, *Phytochemistry*, **1**, 73 (1962).
(40) C. Charaux, *Bull. Soc. Chim. Biol.*, **7**, 1053 (1925).
(41) J. Shinoda and M. Imaida, *Yakugaku Zasshi*, **54**, 107 (1934); *Chem. Abstracts*, **31**, 100 (1937).
(42) T. Kosuge, *Arch. Biochem. Biophys.*, **95**, 211 (1961).
(43) H. J. Klosterman, F. Smith, and C. O. Clagett, *J. Am. Chem. Soc.*, **77**, 420 (1955).
(44) H. J. Klosterman and R. Z. Muggli, *J. Am. Chem. Soc.*, **81**, 2188 (1959).
(45) E. Kodicek and P. W. Wilson, *Biochem. J.*, **76**, 27P (1960).

They suggested that niacytin may possess a chain of β-D-linked D-glucose residues esterified with ferulic acid and sinapic acid. A glycoprotein fraction containing 80–90% of carbohydrate (xylose, arabinose, and galactose) has also been obtained from wheat flour, and there is some evidence suggesting that a xylan esterified with ferulic acid is present in this material.[46] A 3-D-glucosyl derivative of caffeic acid, which can be hydrolyzed by almond emulsin, occurs naturally in the berries of various wild species of tuberous *Solanum*, but not in the berries of the cultivated potato.[38] *Fagopyrum cymosum* contains a compound which has been named shakurichin by Takahashi and Tanabe.[47] One molecule of this compound consists of a D-glucose molecule esterified with *p*-coumaroyl and feruloyl groups, probably on the hydroxyl groups at C-1 and C-6 of the sugar.

Coumarins structurally related to the cinnamic acids also occur widely in plant tissues as D-glucosides. The best known derivative is esculin, found in the horse chestnut. The structure of this glycoside was examined by Head and Robertson[48] and Macbeth,[49] who showed it to be 6-(β-D-glucopyranosyloxy)-7-hydroxycoumarin. The isomeric compound, having the D-glucose residue attached at C-7 (namely, cichoriin) occurs in plant cells.[50] Various other hydroxy and methoxy derivatives of coumarin, existing in D-glucosylated forms, have been described.[3,4]

Stilbene (3) derivatives are normally encountered free in the plant kingdom, but piceatonnol D-glucoside (3′,5-di-D-glucosyloxy-2,4′,6-trihydroxy-3,4-tetramethylenestilbene) has been obtained from the stem bark of *Picea excelsa*.[51] Hathway and Seakins[52] have isolated, from the ether-soluble extractive of *Eucalyptus wandoo* heartwood, 3,4′,5-trihydroxystilbene and the corresponding D-glucoside, which almost certainly has a β-D-glucopyranosyl group attached to the hydroxyl group at C-3.

D-Glucosides of several hydroxyanthraquinones have been reported.[3,4] For example, Schunk and Marchlewski[53] isolated rubiadin D-glucoside from madder root. This compound was later synthesized by Jones and Robertson[54] and shown to be 3-(β-D-glucopyranosyloxy)-1-hydroxy-2-methylanthraquinone.

The last group of D-glucosides to be discussed are the flavonoid deriva-

(46) H. Fausch, W. Kündig, and H. Neukom, *Nature*, **199**, 287 (1963).
(47) K. Takahashi and Y. Tanabe, *Kanazawa Daigaku Yakugakubu Kenkyu Nempo*, **11**, 1 (1961); *Chem. Abstracts*, **56**, 1526 (1962).
(48) F. S. H. Head and A. Robertson, *J. Chem. Soc.*, 2434 (1930).
(49) A. K. Macbeth, *J. Chem. Soc.*, 1288 (1931).
(50) K. W. Merz, *Arch. Pharm.*, **270**, 476 (1932); *Chem. Abstracts*, **27**, 2685 (1933).
(51) W. Grassmann, H. Endres, R. Brockhaus, and K. Merkle, *Chem. Ber.*, **90**, 2416 (1957).
(52) D. E. Hathway and J. W. T. Seakins, *Biochem. J.*, **72**, 369 (1959).
(53) E. Schunk and L. Marchlewski, *J. Chem. Soc.*, **63**, 969 (1893); **65**, 182 (1894).
(54) E. T. Jones and A. Robertson, *J. Chem. Soc.*, 1699 (1930).

tives, which are numerous and ubiquitous in the angiosperms. Thus, luteolin (3′,4′,5,7-tetrahydroxyflavone) has been isolated in three forms, having D-glucosyloxy groups at C-5,[55] C-7,[56] and C-4.[57] Similarly, the flavonol quercetin (3,3′,4′,5,7-pentahydroxyflavone) is found as the 3-,[58] 7-,[58] 4′-,[59] and 3′-*O*-D-glucosyl[60] derivatives. The first three of these compounds are usually known by the trivial names isoquercitrin, quercimeritrin, and spiraeoside, respectively. An unusual flavonol D-glucoside, gossypin, was found in cotton flowers by Rao and Seshadri.[61] This has the structure of 8-D-glucosyloxy-3,3′,4′,5,7-pentahydroxyflavone.

Some flavonol glycosides form derivatives with cinnamic acids. Thus, Hörhammer and coworkers[62] have isolated a *p*-coumaroyl ester (tiliroside) of 3-*O*-D-glucosylkaempferol,[63] and claim that the compound is acylated at the hydroxyl group on C-7. Harborne,[64] however, has shown that it is the D-glucosyl residue which is acylated in tiliroside. If this is the structure, tiliroside structurally resembles other acylated flavonoid glycosides discovered in plants; the majority of these glycosides appear to possess acylated glycosyl groups, and not acyl groups on the flavone nuclei.[64,65]

The most common glycosidic forms of anthocyanidins which occur naturally are the 3-*O*-D-glucosyl and the 3,5-di-*O*-D-glucosyl derivatives. For the aglycon cyanidin (3,3′,4′,5,7-pentahydroxyflavylium), chrysanthemin[66,66a] and cyanin[67,67a,67b] are two such examples. The chrysanthemin and cyanin synthesized by Robinson and coworkers[66a,67b] contained β-D-glucopyranosyl groups, but the present author has found no unequivocal chemical evidence that the corresponding, naturally

(55) G. Barger and F. D. White, *Biochem. J.*, **17,** 836 (1923); H. Nakamura and G. Hukuti, *Yakugaku Zasshi*, **60,** 449 (1940); *Chem. Abstracts*, **34,** 7910 (1940).
(56) S. Hattori and H. Matsuda, *J. Am. Chem. Soc.*, **76,** 5792 (1954); *Acta Phytochim.* (Japan), **15,** 233 (1949); *Chem. Abstracts*, **44,** 3214 (1950).
(57) L. Hörhammer, H. Wagner, and H. S. Dhingra, *Naturwissenschaften*, **45,** 13 (1958); T. Nakaoki and N. Morita, reported by S. Hattori in Ref. 5, p. 324.
(58) G. F. Attree and A. G. Perkin, *J. Chem. Soc.*, 234 (1927); C. D. Douglass, W. L. Howard, and S. H. Wender, *J. Am. Chem. Soc.*, **71,** 2658 (1949).
(59) L. Hörhammer and R. Griesinger, *Naturwissenschaften*, **44,** 427 (1959).
(60) H. L. Hergert and O. Goldschmid, *J. Org. Chem.*, **23,** 700 (1958).
(61) K. V. Rao and T. R. Seshadri, *Proc. Indian Acad. Sci. Sect. A*, **24,** 375 (1946); **25,** 397 (1947).
(62) L. Hörhammer, L. Stich, and H. Wagner, *Naturwissenschaften*, **46,** 358 (1959).
(63) Kaempferol is 3,4′,5,7-tetrahydroxyflavone.
(64) J. B. Harborne, *Phytochemistry*, **3,** 151 (1964).
(65) Reviewed briefly by J. B. Harborne, in Ref. 13, p. 382.
(66) R. Willstätter and E. K. Bolton, *Ann.*, **412,** 136 (1916).
(66a) S. Murkami, A. Robertson, and R. Robinson, *J. Chem. Soc.*, 2665 (1931).
(67) R. Willstätter and A. E. Everest, *Ann.*, **401,** 189 (1913).
(67a) G. M. Robinson and R. Robinson, *Nature*, **128,** 413 (1931).
(67b) R. Robinson and A. R. Todd, *J. Chem. Soc.*, 2488 (1932).

occurring compounds have the same D-glucosidic configurations. Here again, however, circumstantial evidence suggests that the synthetic and naturally occurring compounds are identical. A similar state of affairs exists with many other anthocyanins.

Mono-*O*-D-glucosylanthocyanidins may also possess other D-glycosyl groups on the flavonoid nucleus. *Papaver* species, for example, contain pelargonidin (3,4′,5,7-tetrahydroxyflavylium) having a D-glucosyloxy group at C-7 and a sophorosyloxy[68] group at C-3; cyanidin is also found in this genus in the same glycosidic form.[69] 5-D-Glucosyl groups are accompanied by 3-L-rhamnosyl groups on pelargonidin, cyanidin, and peonidin (3,4′,5,7-tetrahydroxy-3′-methoxyflavylium) in *Lathyrus* spp.[69]

Chalcones (**11**) and aurones (**13**), the so-called anthochlor pigments, appear to be confined only to a few plant families, in particular, to the Compositae and Leguminosae. *Coreopsis* and *Cosmos* spp. contain the 4′-D-glucoside of butein (2′,3,4,4′-tetrahydroxychalcone),[70] and 4′-D-glucosyloxy-2′,4-dihydroxychalcone is located in the flowers of gorse (*Ulex europaeus*)[71] and *Dahlia variablis*.[72] Phloridzin is, perhaps, the best known dihydrochalcone (**12**); it was isolated in 1835 from the root bark of the apple tree by de Koninck.[73] The compound has been well characterized by Robertson and coworkers,[74] who showed that its structure is that of 2′-β-D-glucopyranosyloxy-4′,6′-dihydroxy-β-phenylpropiophenone.

The known 2-benzalcoumaranones [aurones (**13**)] are few. Aureusin and cernuoside, two isomeric D-glucosides of aureusidin (namely, 3,3′,4,4′-tetrahydroxyaurone) have been shown by Geissman and Harborne[75] to be the 6- and 4-*O*-D-glucosyl derivatives, respectively. Sulphurein, an aurone found in *Cosmos sulphureus*, is 6-D-glucosyloxy-3′,4′-dihydroxyaurone.[70a]

(*ii*) D-*Galactose*.—Mono-*O*-D-galactosyl derivatives of phenols are much less frequently encountered in Nature than are *O*-D-glucosyl derivatives. D-Galactose esters are unknown, but several aryl D-galactosides in the flavonoid series have been reported. From the point of view of the carbohydrate structure, the only D-galactoside that appears to have been examined in any detail is the 3-*O*-β-D-galactopyranosyl derivative of cyanidin, which occurs in the cacao bean. This derivative was synthesized by Forsyth

(68) Sophorosyloxy is 2-*O*-β-D-glucopyranosyl-D-glucopyranosyloxy.
(69) J. B. Harborne, *Phytochemistry*, **2**, 85 (1963).
(70) T. A. Geissman, *J. Am. Chem. Soc.*, **64**, 1704 (1942).
(70a) M. Shimokoryama and S. Hattori, *J. Am. Chem. Soc.*, **75**, 1900 (1953).
(71) J. B. Harborne, *Phytochemistry*, **1**, 203 (1962).
(72) C. G. Nordström and T. Swain, *Arch. Biochem. Biophys.*, **60**, 329 (1956).
(73) L. de Koninck, *Ann. Pharm.*, **15**, 75, 258 (1835).
(74) F. R. Johnson and A. Robertson, *J. Chem. Soc.*, 21 (1930). A. Müller and A. Robertson, *ibid.*, 1170 (1933).
(75) T. A. Geissman and J. B. Harborne, *J. Am. Chem. Soc.*, **77**, 4622 (1955).

and Quesnel,[76] and the properties of the synthetic and isolated compounds were compared. In particular, the rates of enzymic hydrolysis of the two compounds were measured, and shown to be identical within the limits of experimental error. A 3-*O*-D-galactosyl derivative of delphinidin (3,3′,4′,5,5′,7-hexahydroxyflavylium) occurs in *Empetrum nigrum*,[77] and the occurrence of other *O*-D-galactosyl derivatives of anthocyanidins in other plant species is mentioned by Harborne.[13] Examples of naturally occurring flavonol 3-D-galactosides are hyperin, a derivative of quercetin found in several plants including *Rumex acetosa*,[78] and trifolin, the corresponding kaempferol derivative, which may be isolated from *Trifolium pratense*.[79] Chaerophyllin is a mono-D-galactoside of the flavone luteolin. The position of the D-galactosyl residue in this compound is not yet certain, however.[80]

(*iii*) D-*Fructose*.—Only one example of this sugar in combination with a phenol is known. The glycoside, named pajaneelin, is found in relatively high concentrations in the bark of the tree *Pajaneelia rheedi*. Pajaneelin is D-fructosyl *p*-coumarate, but nothing is yet known of the ring size of the hexose residue or the configuration of the anomeric carbon atom.[81]

(*iv*) D-*Glucuronic Acid*.—D-Glucuronic acid derivatives of phenols were detected in plants as early as 1901, when Molisch and Goldschmiedt[82] discovered scutellarin, a D-glucosiduronic acid derivative of scutellarein (4′,5,6,7-tetrahydroxyflavone) in the leaves of *Scutellaria altissima*. Later, Shibata and coworkers[83] isolated baicalin from *Scutellaria baicalensis*; it was shown to be a 7-D-glucopyranosyluronic acid derivative of 5,6,7-trihydroxyflavone.[84] Marsh,[85] using β-D-glucuronidase (β-D-glucosiduronic acid D-glucuronohydrolase) obtained evidence that the D-glucuronic acid residues in both baicalin and scutellarin have the β-D configuration. Apigenin (4′,5,7-trihydroxyflavone), substituted at C-7 with D-glucuronic acid, has also been isolated, but the detailed structure of the glycosyl

(76) W. G. C. Forsyth and V. C. Quesnel, *Biochem. J.*, **65**, 177 (1957).
(77) K. Hayashi, G. Suzushino, and K. Ouchi, *Proc. Japan Acad.*, **27**, 430 (1951); *Chem. Abstracts*, **46**, 7567 (1952).
(78) L. Hörhammer and E. Volz., *Arch. Pharm.*, **288**, 58 (1955).
(79) S. Hattori, M. Hasegawa, and M. Shimokoriyama, *Acta Phytochim.* (Japan), **13**, 99 (1943); *Chem. Abstracts*, **41**, 5156 (1951).
(80) G. R. Clemo and D. G. I. Felton, *J. Chem. Soc.*, 1535 (1949).
(81) A. Kameswaramma and T. R. Seshadri, *Proc. Indian Acad. Sci. Sect. A*, **25**, 43 (1947).
(82) H. Molisch and G. Goldschmiedt, *Monatsh.*, **22**, 679 (1901); G. Goldschmiedt and E. Zerner, *ibid.*, **31**, 439 (1910).
(83) K. Shibata, S. Iwata, and M. Nakamura, *Acta Phytochim.* (Japan), **1**, 105 (1923).
(84) K. Shibata and S. Hattori, *Acta Phytochim.* (Japan), **5**, 117 (1930); *Chem. Abstracts*, **25**, 1528 (1931).
(85) C. A. Marsh, *Nature*, **176**, 176 (1955).

group is not yet known.[86] *Scutellaria galericulata* leaves contain a β-D-glucopyranosiduronic acid of chrysin (5,7-dihydroxyflavone), the configuration of the uronic acid moiety again having been deduced enzymically.[87]

Pearl and Darling[88] have isolated, from *Populus grandidentata* leaves, a flavonol D-glucosiduronic acid identical in all respects with a compound obtained by Endres and coworkers[89] and March[85] from *Phaseolus vulgaris* and with the known quercetin 3-D-glucosiduronic acid found by Sasaki and Watanabe in *Gaultheria miqueliana*.[90]

(*v*) L-*Rhamnose*.—Several examples of aryl L-rhamnosides occur in Nature, particularly in the flavonol series. However, there have been virtually no studies of the anomeric configuration and ring size of the L-rhamnosyl groups in these compounds. It is generally assumed, by analogy with other naturally occurring L-rhamnose derivatives in plants, that the sugar assumes the L-pyranoid form, and there is as yet no reason to doubt this assumption. In the naturally occurring cardiac glycosides, the L sugars are normally bonded by α-L-glycosidic linkages,[91] and, in the known L-rhamnose-containing disaccharide glycosides of phenols, the L-rhamnose residue has this configuration at C-1. It, therefore, seems probable that most of the mono-L-rhamnosyl derivatives of plant phenols have the α-L configuration also; this may not be true in all cases, however. For example, Suzuki[92] was unable to hydrolyze myricitrin (3′,4′,5,5′,7-pentahydroxy-3-L-rhamnosyloxyflavone) with an enzyme preparation (rhamnodiastase) from *Rhamnus dahurica*; the enzyme possessed α-L-rhamnosidase (α-L-rhamnoside rhamnohydrolase) activity.

The naturally occurring aryl L-rhamnosides include quercitrin, namely, quercetin substituted at C-3. This is a fairly common derivative in plant tissues.[3–5] Mono-L-rhamnosides of kaempferol, in which substitution occurs at the hydroxyl groups at C-3 and C-7, are known,[93,94] and kaempferitrin (lespedin, 3,7-di-L-rhamnosylkaempferol) has also been reported.[95]

(86) K. Imai and T. Mayama, *Yakugaku Zasshi*, **73,** 131 (1953); *Chem. Abstracts*, **47,** 12370 (1953).
(87) C. A. Marsh, *Biochem. J.*, **59,** 58 (1955).
(88) I. A. Pearl and S. F. Darling, *J. Org. Chem.*, **28,** 1442 (1963).
(89) G. Endres, R. Hüttel, and L. Kaufmann, *Ann.*, **537,** 205 (1939).
(90) T. Sasaki and Y. Watanabe, *Yakugaku Zasshi*, **76,** 892 (1956); *Chem. Abstracts*, **51,** 2766 (1957).
(91) W. Klyne, *Biochem. J.*, **47,** xli (1950).
(92) H. Suzuki, *Arch. Biochem. Biophys.*, **99,** 476 (1962).
(93) F. E. King and R. M. Acheson, *J. Chem. Soc.*, 168 (1950).
(94) R. G. Cooke and H. F. Haynes, *Australian J. Chem.*, **13,** 150 (1960).
(95) A. G. Perkin, *J. Chem. Soc.*, **91,** 435 (1907). M. Hasegawa, *Acta Phytochim.* (Japan), **11,** 299 (1940); *Chem. Abstracts*, **35,** 1403 (1941).

In rare instances, 3-L-rhamnosyl derivatives of anthocyanidins occur in plant tissues.[69,96] Frangulin (franguloside), an L-rhamnoside of 4,5,7-trihydroxy-2-methylanthraquinone, is found in *Rhamnus* spp.[97]; it probably[98] has the glycosyloxy group at C-7.

(*vi*) D-*Xylose.*—Only two references appear to have been made in the literature to aryl mono-D-xylosides,[99] although D-xylose is fairly common, in the combined form, in disaccharide glycosides. In both references, the natural occurrence of 3-D-xylosylquercetin is noted, but no other details of structure are given.

(*vii*) L-*Arabinose.*—L-Arabinosides of plant phenols are not common, but this pentose occurs as the α-L-pyranoside and α-L-furanoside forms, and a β-L-arabinoside may also exist.

In 1940, Ohta[100] isolated a 3-L-arabinosyl derivative of quercetin (avicularin) from *Polygonum aviculare.* Hörhammer and coworkers[101] showed that this compound has a strongly positive, specific optical rotation and that the glycosidic linkage is cleaved by almond emulsin. In view of this evidence, they suggested that avicularin is an α-L-arabinoside. The same compound was isolated from *Psidium guaijava* leaves by El Khadem and Mohammed,[102] and, by methylation, it was shown that the L-arabinose residue is furanoid. Also found in *P. guiajava* is a second glycoside of quercetin, guaijaverin, which has been clearly shown by degradation and synthesis to be 3-(α-L-arabinopyranosyloxy)-3′,4′,5,7-tetrahydroxyflavone.[102] It has been suggested that polystachoside, $[\alpha]_D$ $-25.9°$, is a 3-β-L-arabinosyl derivative of quercetin, found in *Polygonum polystachyum.* This conclusion, however, is again only based on enzymic hydrolysis studies.[101]

Other aryl L-arabinosides whose detailed structures are unknown include a 3-substituted kaempferol, found in horse chestnut,[103] and a 4′-L-arabinosyl 3-L-rhamnosyl derivative of the same flavonol.[104] Various 3-L-arabinosyl derivatives of anthocyanidins occur in rare instances.[105,106]

(96) M. Shimokoriyama, *Botan. Mag.* (Tokyo), **62,** 737 (1949).
(97) M. Bridel and C. Charaux, *Bull. Soc. Chim. Biol.*, **15,** 642 (1933).
(98) O. Schindler, *Helv. Chim. Acta,* **29,** 411 (1946).
(99) H. W. Siegelman, *J. Biol. Chem.*, **213,** 647 (1955); T. Nakaoki and N. Morita, *Yakugaku Zasshi,* **76,** 323 (1956); *Chem. Abstracts,* **50,** 9686 (1956).
(100) T. Ohta, *Z. Physiol. Chem.*, **263,** 221 (1940).
(101) L. Hörhammer, R. Hansel, G. Kriesmair, and W. Endres, *Arch. Pharm.*, **268,** 419 (1955).
(102) H. El Khadem and Y. S. Mohammed, *J. Chem. Soc.*, 3320 (1958).
(103) H. J. Germann, L. Endres, R. Cobet, and U. Fielder, *Naturwissenschaften,* **42,** 181 (1955).
(104) L. Hörhammer, L. Endres, H. Wagner, and F. Richthammer, *Arch. Pharm.*, **290,** 342 (1957).
(105) M. Metche and E. Urion, *Compt. Rend.*, **252,** 356 (1961).
(106) J. B. Harborne, *Arch. Biochem. Biophys.*, **92,** 171 (1962).

b. Disaccharide Components.—Aryl diglycosides are quite common in plant tissues, and disaccharides acylated with a phenolic acid at the anomeric carbon atom of the reducing end-group have also been isolated. The better known disaccharides found in these combined forms usually contain at least one D-glucose residue per molecule, and, with few exceptions, it is this residue which is bonded by a β-D linkage to the phenol residue. Where D-glucose is absent from the disaccharide, a D-galactose residue is normally attached to the phenol residue. Only in rare instances are other monosaccharide residues directly involved in bonding to the aryl aglycon.

(*i*) *Sophorose* (*2*-O-β-D-*Glucopyranosyl*-D-*glucopyranose*).—The pods of *Sophora japonica* contain a kaempferol sophoroside which was the first known source of this disaccharide. The pigment was originally isolated by Rabaté and Dussy[107]; Rabaté[108] later showed that the hydroxyl group at C-3 of the flavonol is substituted by the sophorose residue. Freudenberg and coworkers[109] and Clancy[110] have confirmed the identity of the sugar, and substantiated its structure by preparation of the crystalline disaccharide (and its octaacetate) and by periodate oxidation studies.

Harborne[111] has reviewed the occurrence of sophorose in combination with phenols, and has concluded that *O*-sophorosyl groups are the commonest *O*-diglycosyl groups in the flavonoid series. Five anthocyanin pigments and at least ten flavonol derivatives are known, all of which contain a sophorose residue. Sophorosides of anthocyanidins and flavonols have not, apparently, yet been found together in the same plant species.

(*ii*) *Gentiobiose* (*6*-O-β-D-*Glucopyranosyl*-D-*glucopyranose*).—This disaccharide occurs only rarely in combination with plant phenols. In *Primula sinensis*, the 3-gentiobiosides of pelargonidin, cyanidin, quercetin, and kaempferol are found,[112] and *Tritonia* flowers also appear to contain gentiobiosylpelargonidin.[69] Gentiobiosyl caffeate, which occurs in *Petunia hybrida*,[38] is the only example of a naturally occurring ester of this disaccharide.

For the compounds mentioned above, the gentiobiose has not been rigorously characterized. At best, the disaccharide, liberated by partial acid hydrolysis, has only been identified by paper chromatography using several solvent systems (with known D-glucose disaccharides for comparison). Further studies are obviously necessary in order to confirm or refute the identity of gentiobiose in these natural products.

(107) J. Rabaté and J. Dussy, *Bull. Soc. Chim. Biol.*, **20**, 459, 467 (1938).
(108) J. Rabaté, *Bull. Soc. Chim. France*, **7**, 565 (1940).
(109) K. Freudenberg, H. Knauber, and F. Cramer, *Chem. Ber.*, **84**, 144 (1951).
(110) M. J. Clancy, *J. Chem. Soc.*, 4213 (1960).
(111) J. B. Harborne, *Experientia*, **19**, 7 (1963).
(112) J. B. Harborne and H. S. A. Sherratt, *Biochem. J.*, **78**, 298 (1961).

(*iii*) *Maltose* (*4*-O-α-D-*Glucopyranosyl*-D-*glucopyranose*).—Maltose is said to be present as a cyanohydrin in combination with 2′,4′,5,7-tetrahydroxyflavone, in lotusin, isolated from the poisonous herb *Lotus arabicus* by Dunstan and Henry.[113] These workers, however, present little evidence to suggest that the combined sugar was, in fact, maltose.

(*iv*) O-*Galactosylgalactose*.—Birch leaves contain myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) substituted at the hydroxyl group at C-3 with a D-galactose disaccharide of unknown constitution.[114]

(*v*) *Neohesperidose* (*2*-O-α-L-*Rhamnopyranosyl*-D-*Glucopyranose*).—Three flavanones (**7**) are now known to occur, as derivatives of this disaccharide, in the peel of *Citrus* spp. These compounds are naringin, neohesperidin, and poncirin, which are the 7-*O*-β-neohesperidosyl derivatives of 4′,5,7-trihydroxyflavanone, 3′,5,7-trihydroxy-4′-methoxyflavanone, and 5,7-dihydroxy-4′-methoxyflavanone, respectively. The nature of the sugar moiety in these compounds has been carefully checked by methylation studies, and the glycosidic linkages have been determined by the use of enzymes and by examination of molecular rotation data.[115] The work of Horowitz and Gentili[116] has corrected the original idea that naringin contains an *O*-(4-*O*-L-rhamnosyl-D-glucosyl) group.

(*vi*) *Rutinose* (*6*-O-α-L-*Rhamnopyranosyl*-D-*glucopyranose*).—This disaccharide frequently occurs in combination with plant phenols. For many years, it was believed that the L-rhamnose was in the β-L configuration[117]; work by Gorin and Perlin[118] has shown that it is actually the α-L form.

Rutin (3-*O*-β-rutinosylquercetin) is one of the major flavonoid glycosides, first found in *Ruta graveoleus*.[119] It has since been reported to occur in several different organs of many plant species.[120] 3-*O*-Rutinosyl derivatives of cyanidin and pelargonidin have been detected in several plants, including *Poinsettia*[121] and *Cestrum*.[13] Morindin, a substituted anthraquinone from *Coprosmo australis*, appears to be 5,6-dihydroxy-1,2-dimethoxyanthraquinone having[122] an *O*-rutinosyl group at C-6. Rutinosyl *p*-coumarate is an example of an ester, found in *Lathyrus odoratus* by Harborne and Corner.[38]

(113) W. R. Dunstan and T. A. Henry, *Phil. Trans. Roy. Soc. London Ser. B*, **67**, 224 (1901).
(114) L. Hörhammer, H. Wagner, and R. Luck, *Arch. Pharm.*, **290**, 338 (1957).
(115) R. M. Horowitz and B. Gentili, *Tetrahedron*, **19**, 773 (1963).
(116) G. Zemplén and A. K. Tettamanti, *Ber.*, **71**, 2511 (1938).
(117) G. Zemplén and A. Gerecs, *Ber.*, **67**, 2049 (1934); **68**, 1318 (1935).
(118) P. A. J. Gorin and A. S. Perlin, *Can. J. Chem.*, **37**, 1930 (1959).
(119) A. Weiss, *Pharm. Centralbl.*, **13**, 305 (1842).
(120) Ref. 3, p. 614; Ref. 4, p. 583; Ref. 5, p. 338.
(121) S. Asen, *Plant Physiol.*, **33**, 14 (1958).
(122) L. H. Briggs and P. W. le Quesne, *J. Chem. Soc.*, 3471 (1963).

New *O*-L-rhamnosyl-D-glucose derivatives of phenols isolated are often automatically assumed to be rutinosides. This assumption should be discouraged, particularly in view of the discovery of neohesperidose, and the likelihood that other *O*-L-rhamnosyl-D-glucosides may exist in Nature.

(*vii*) O-*Glucosylrhamnose.*—Seebeck and Schindler[123] isolated, from *Rhamnus frangula*, an unusual disaccharide glycoside which they called glucofrangulin. On complete hydrolysis, this material yielded glucose, rhamnose, and emodin (4,5,7-trihydroxy-2-methylanthraquinone), and, on treatment with the enzyme rhamnodiastase, gave glucose and frangulin (*O*-rhamnosylemodin). From the results of alkaline hydrolysis studies, they concluded that the diglycosyl group is α-D-linked to the hydroxyl group at C-7 of the quinone.[98]

(*viii*) *Robinobiose* (*6*-O-α-L-*Rhamnopyranosyl*-D-*galactopyranose*).—The flavonol glycoside robinin occurs in the flowers of *Robinia pseudacacia*[124] and in several other plant species. Originally, it was believed that robinin was a trisaccharide glycoside, as Charaux[125,126] claimed that hydrolysis with rhamnodiastase yielded kaempferol and a sugar, one molecule of which was comprised of 1 residue of galactose and 2 rhamnose residues. Later work by Zemplén and coworkers[117,127] showed that this constitution was incorrect, and that the structure of robinin is 7-*O*-L-rhamnosyl-3-*O*-(*O*-L-rhamnosyl-D-galactosyl)kaempferol. This result was supported by the results of independent studies of Shimokoriyama.[96] Early workers had believed that, as with rutinose, a β-L-rhamnosyl residue was present in the disaccharide.[128] Gorin and Perlin,[118] however, have shown that the 6-deoxyhexose has the α-L configuration.

(*ix*) O-*Rhamnosylrhamnose.*—Quercetin and kaempferol glycosides, which readily yield 2 moles of rhamnose per mole when hydrolyzed with formic acid, have been found in the foliage of *Exocarpus cupressiformis.* The suspicion that a rhamnose disaccharide is glycosidically bonded to the hydroxyl group at C-3 in these flavonols has been confirmed by methylation, but the structure of the disaccharide has not yet been deduced.[94]

(*x*) *Sambubiose* (*2*-O-β-D-*Xylopyranosyl*-D-*glucopyranose*).—This disaccharide is believed to occur fairly commonly as a diglycosyl group in anthocyanins. An *O*-D-xylosyl-D-glucoside of cyanidin, detected in *Sambucus nigra*, was originally believed to be a 3-primveroside[129] (see p. 390),

(123) E. Seebeck and O. Schindler, *Helv. Chim. Acta*, **29**, 317 (1946).
(124) C. Zwenger and F. Dronke, *Ann.*, **Suppl. 1**, 263 (1861).
(125) C. Charaux, *Compt. Rend.*, **178**, 1312 (1924).
(126) C. Charaux, *Bull. Soc. Chim. Biol.*, **8**, 915 (1926).
(127) G. Zemplén and R. Bognár, *Ber.*, **74**, 1783 (1941).
(128) G. Zemplén, A. Gerecs, and H. Flesch, *Ber.*, **71**, 774 (1938).
(129) L. Reichel, H. Stroh, and W. Reichwald, *Naturwissenschaften*, **44**, 468 (1957).

but it was later shown to be a 3-sambubioside.[130] The structure of the combined disaccharide has, however, only been deduced by methylation, hydrolysis, and examination of the resulting, partially methylated products on paper chromatograms; the β-D linkage was indicated by its hydrolysis with almond emulsin. Pelargonidin 3-sambubioside is believed to be present in *Streptocarpus*, but, here again, the identity of the disaccharide moiety is based mainly on chromatographic and electrophoretic evidence.[69]

(*xi*) *Primverose* (*6*-O-β-D-*Xylopyranosyl*-D-*glucopyranose*).—The existence of this disaccharide in combination with plant phenols has been known for many years. Primverose is probably less abundant than sambubiose. It occurs in the glycoside primverin which was isolated in 1909 from *Primula officinalis*.[131] The glycoside is the 2-*O*-β-primverosyl derivative of methyl 2-hydroxy-4-methoxybenzoate, as was proved by degradation[132] and synthesis.[133] A structurally related compound is monotropitin, which was isolated by Bridel[134] from *Monotropa hypopitys*. This compound and gaultherin (isolated by Bourquelot from a different source[135]) have the same structure, namely, methyl 2-β-primverosyloxybenzoate. The glycoside has been synthesized by Robertson and Waters.[136] Other naturally occurring phenolic primverosides include fabiatrin, a β-primveroside of scopoletin (6-hydroxy-7-methoxycoumarin),[137] and galiosin, a 1-*O*-β-primverosyl derivative of pseudopurpurin (1,2,4-trihydroxyanthraquinone-3-carboxylate) found in the madder plant. This latter compound is probably accompanied by the 3-*O*-β-primverosyl derivative of rubiadin (1,3-dihydroxy-2-methylanthraquinone), although the evidence for this structure is not so convincing as for galiosin.[138]

(*xii*) *Lathyrose* (O-β-D-*Xylosyl*-D-*galactose*).—Peonidin, cyanidin, and pelargonidin are substituted at C-3 with *O*-(*O*-β-D-xylosyl-D-galactosyl) residues in three glycosides occurring in *Lathyrus odoratus* flowers. Partial acid hydrolysis of these three glycosides yields the corresponding anthocyanidin D-galactosides and D-xylose, together with D-galactose and an oligosaccharide which is, presumably *O*-D-xylosyl-D-galactose.[69] The latter is hydrolyzed by almond emulsin, suggesting that the D-xylose residue

(130) L. Reichel and W. Reichwald, *Naturwissenschaften*, **47,** 40 (1960).
(131) A. Goris and M. Mascré, *Compt. Rend.*, **149,** 947 (1909).
(132) A. Goris and C. Vischniac, *Compt. Rend.*, **169,** 871, 975 (1919).
(133) E. T. Jones and A. Robertson, *J. Chem. Soc.*, 1618 (1933).
(134) M. Bridel, *Compt. Rend.*, **177,** 652 (1923); **179,** 991 (1924); **180,** 1421 (1925).
(135) E. Bourquelot, *J. Pharm. Chim.*, **3,** 577 (1896).
(136) A. Robertson and R. B. Waters, *J. Chem. Soc.*, 1881 (1931).
(137) G. E. Edwards and H. Rogerson, *Biochem. J.*, **21,** 1010 (1927); D. N. Chaudhury, R. A. Holland, and A. Robertson, *J. Chem. Soc.*, 1671 (1948).
(138) R. Hill and D. Richter, *J. Chem. Soc.*, 1714 (1936).

has the β-D configuration.[64] The name lathyrose was suggested by Harborne[13] for this disaccharide.

(*xiii*) *Vicianose* (*6*-O-β-L-*Arabinopyranosyl*-D-*glucopyranose*).—Vicianose is a rare disaccharide which occurs in a combined form in the glycoside gein. *Geum urbanum* contains gein, and it was first isolated from this source by Bourquelot and Hérissey[139] in 1905. The discovery that gein is a β-vicianoside of eugenol came from later studies by Hérissey and Cheymol,[140] and this conclusion was confirmed by synthesis.[141] Vicianose is also found, in combination with methyl salicylate, in *Vio'a cornuta*. This glycoside, which was been named violutoside, was isolated by Picard[142] and synthesized by Robertson and Waters.[143]

(*xiv*) *2*-O-D-*Apiofuranosyl*-D-*glucopyranose*.—This is a rare disaccharide which occurs in apiin, a derivative of apigenin (4′,5,7-trihydroxyflavone) found in *Petroselinum crispum*, *Vicia hirsuta*, and a number of other plants. The glycoside has been the subject of a great deal of work, reviewed, up to 1948, by Hudson.[144] The (1→2)-linkage between the D-apiose and the D-glucose residues was deduced by methylation studies; and partial hydrolysis of apiin yields 7-*O*-β-D-glucopyranosylapigenin.[145] These results, in conjunction with the report that naturally occurring apiose has the D configuration,[146] suggest that apiin is 7-*O*-(2-*O*-D-apiofuranosyl-β-D-glucopyranosyl)apigenin. The anomeric configuration of the D-apiofuranosyl residue has yet to be determined.

Luteolin 7-apiosylglucoside is also present in *Petroselinum crispum*.[147] No details of the fine structure of this compound are known, but it may well have the same glycosidic moiety as apiin.

(*xv*) *6*-O-D-*Apiofuranosyl*-D-*glucopyranose*.—*Viburnum furcatum* leaves contain furcatin, a glycoside of *p*-vinylphenol which, from hydrolysis and periodate oxidation studies, appears to be an *O*-(6-*O*-D-apiofuranosyl-β-D-glucopyranosyl) derivative.[148]

The D-configuration for the apiose residue in this disaccharide is suggested by the present author on the basis of the work of Gorin and Perlin.[146] As this structure is derived mainly from periodate oxidation data, it

(139) E. Bourquelot and H. Hérissey, *Compt. Rend.*, **140**, 870 (1905).
(140) H. Hérissey and J. Cheymol, *Compt. Rend.*, **180**, 384 (1925); **181**, 565 (1925).
(141) D. N. Chaudhury and A. Robertson, *J. Chem. Soc.*, 2054 (1949).
(142) P. Picard, *Compt. Rend.*, **182**, 1167 (1926).
(143) A. Robertson and R. B. Waters, *J. Chem. Soc.*, 2970 (1932).
(144) C. S. Hudson, *Advan. Carbohydrate Chem.*, **4**, 57 (1949).
(145) R. Hemming and W. D. Ollis, *Chem. Ind.* (London), 85 (1953).
(146) P. A. J. Gorin and A. S. Perlin, *Can. J. Chem.*, **36**, 480 (1958).
(147) C. G. Nordström, T. Swain, and A. J. Hamblin, *Chem. Ind.* (London), 58 (1953).
(148) S. Hattori and H. Imaseki, *J. Am. Chem. Soc.*, **81**, 4424 (1959).

should, perhaps, be accepted with caution, particularly as little is known about the oxidation of oligosaccharides containing nonreducing apiosyl end-groups, and because Hattori and Imaseki[148] only estimated the total acid produced. In theory, formic acid and a complex carboxylic acid of high molecular weight should be formed by the oxidation of furcatin. No information regarding the configuration of the glycosidic linkage in the disaccharide is as yet available.

c. Trisaccharide Components.—Only during the past two to three years has it become clear that trisaccharide glycosides of phenols occur in plant tissues, although complex tri- and tetra-saccharide glycosides in the alkaloid series were reported[149] in 1955 and 1957. An examination of the phenolic derivatives has, so far, only been superficial and in general has been limited to hydrolyses in conjunction with paper-chromatographic and electrophoretic studies.

(*i*) O-*Glucosyl*-O-*glucosylglucoses.*—Two different linear glucose trisaccharides in combination with flavonoids are known, although definite evidence of structure is very sparse for both. For example, wild and cultivated varieties of *Pisum* contain triglucosides of kaempferol and quercetin, and, in both instances, the triglycosyl group is acylated with a cinnamic acid derivative. Harborne[111] states that the combined trisaccharide has at least one, and probably two, β-D-(1→2) linkages. This might be expected on biosynthetic grounds, as these pigments coexist in the tissues with kaempferol 3-sophoroside and quercetin 3-sophoroside.

In *Primula sinensis*, several flavonol 3-triglucosides and anthocyanidin 3-triglucosides are found. Gentiobiosides occur in the same plant, and, consequently, it has been suggested[13,112] that the two linkages in the trisaccharide are β-D-(1→6). A similar trisaccharide has been reported to occur in wheat germ; the aglycon is 2-methoxyquinol, with *O*-glycosylation occurring *meta* to the methoxyl group.[150]

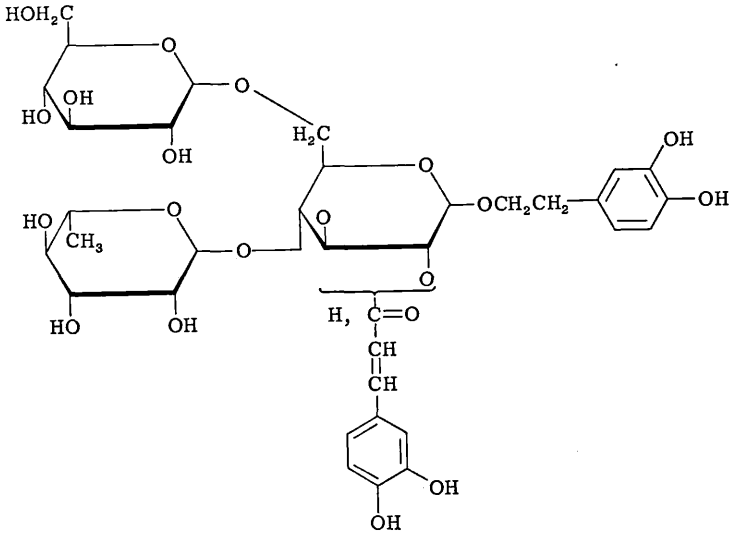
(*ii*) *Glucose, Rhamnose, and Galactose.*—Tappi and Menziani[151] have isolated, from the pollen of *Lilium candidum*, a flavonoid glycoside which, on hydrolysis of one molecule yields 1 molecule each of isorhamnetin (3,4′,5,7-tetrahydroxy-7-methoxyflavone), glucose, rhamnose, and galactose. Methylation clearly showed that only the 3-hydroxyl group of the flavonoid is substituted, and, therefore, a triglycosyl group composed of the above three sugars must be present in the molecule. The monosaccharide sequence is unknown.

(*iii*) O-β-D-*Glucopyranosyl*-(*1→2*)-O-[α-L-*rhamnopyranosyl*-(*1→6*)]-β-D-

(149) R. Kuhn, I. Löw, and H. Trischmann, *Chem. Ber.*, **88**, 1492, 1670 (1955); **90**, 203 (1957).
(150) J. Conchie, A. Moreno, and C. E. Cardini, *Arch. Biochem. Biophys.*, **94**, 342 (1961).
(151) G. Tappi and E. Menziani, *Gazz. Chim. Ital.*, **85**, 694 (1955).

glucopyranose.—Preliminary studies suggest that this branched-chain oligosaccharide is a substituent of cyanidin in *Begonia* and *Rubus idaeus.*[152] Evidence of structure is derived from the fact that the pigment, on complete hydrolysis with acid, yields cyanidin, D-glucose, and L-rhamnose in the molecular ratios of 1:2:1. Partial hydrolysis with acid produces cyanidin 3-sophoroside and cyanidin 3-rutinoside, and oxidative degradation of the cyanidin nucleus with hydrogen peroxide liberates an oligosaccharide which behaves as a trisaccharide on paper chromatograms. This sugar is cleaved by β-D-glucosidase, with the formation of rutinose and D-glucose.

(*iv*) O-L-*Rhamnopyranosyl-*(*1→4*)-O-[D-*glucopyranosyl-*(*1→6*)]-D-*glucopyranose.*—Stoll, Renz, and Brack[153] have isolated a material, echinacoside, from the root of *Echinacea angustifolia.* The compound has been methylated and hydrolyzed. From the results, it was given the partial structure (21).



(21)

(*v*) O-β-D-*Xylopyranosyl-*(*1→2*)-O-[α-L-*rhamnopyranosyl-*(*1→6*)]-β-D-*glucopyranose.*—*Begonia* flowers contain cyanidin *O*-glycosylated on the hydroxyl group at C-3 with a branched chain *O*-xylosylrutinose derivative.[152] Evidence for structure was again obtained by methods similar

(152) J. B. Harborne and E. Hall, *Biochem. J.*, **88**, 41P (1963).
(153) A. Stoll, J. Renz, and A. Brack, *Helv. Chim. Acta*, **33**, 1877 (1950).

to those used with the above D-glucosylrutinose. In this instance, partial hydrolysis gave cyanidin 3-rutinoside and cyanidin 3-sambubioside. These two pigments, on treatment with hydrogen peroxide, yield rutinose and sambubiose, respectively.

Other trisaccharides which, it has been suggested, occur in combination with flavonoids in plants are *O*-β-D-glucopyranosyl-(1→2)-*O*-α-L-rhamnopyranosyl-(1→6)-D-glucopyranose as a kaempferol derivative in potato flowers,[111,154] and a galactosylsophoroside of kaempferol in *Pisum arvense*.[111] However, for both, a great deal more work is required before these structures can be fully accepted.

III. Methods

This Section is mainly concerned with the procedures available for elucidation of the structures of the carbohydrate residues bonded to phenols. In some instances, it is important to know something of the structure of the phenol itself in order to determine the nature of the glycosyl residue.

1. *Isolation*

The methods used for the isolation of phenol–carbohydrate derivatives from plant tissues are extremely varied, and will not be given detailed treatment here. The care required to prevent degradation in the initial stages of extraction does need to be emphasized, however. The main dangers arise from incomplete inactivation of hydrolytic (and, to a lesser extent, oxidative) enzyme systems, both of which may remove glycosyl residues from phenolic compounds. In order to prevent this degradation, it is best to ensure that whole plants that have been removed from their natural environment, or organs that have been excised from a plant, are immediately killed by immersion in hot alcohol or other suitable solvent. The tissues should then be quickly broken down by mechanical means. It should be remembered that many carbohydrases are quite stable proteins which maintain their catalytic function in the presence of relatively high concentrations of cold, organic solvents. More-specific protein-denaturing reagents, such as aqueous mercuric chloride solution, might perhaps successfully replace organic solvents in some cases; these would be useful with heat-labile substances.

High temperatures and weakly acidic solutions favor the degradation

(154) J. B. Harborne, *Biochem. J.*, **84**, 100 (1962).

of many glycosides, particularly glycofuranosides, and high pH values and oxidizing agents may rapidly produce changes in compounds having *o*- or *p*-dihydroxyl groups, even if one of the hydroxyl groups is glycosylated.

The isolation procedures that have been used for naturally occurring phenol–carbohydrate derivatives include such classical methods as extraction with a solvent and precipitation of lead salts, but column and paper chromatography have had the greatest impact in this field. Since the introduction of partition chromatographic methods for naturally occurring phenolic compounds,[155] there has been remarkable progress, particularly in the study of flavonoid glycosides.[156] Thin-layer chromatography of phenolic compounds has been developed, and such excellent new adsorbents as polyamide[157] have been introduced. A useful procedure complementary to chromatography is paper electrophoresis.[158]

2. *Determination of Structure*

The procedures used for the elucidation of the structures of glycosyl residues are largely based on the general methods of carbohydrate chemistry. For example, when a monosaccharide or an oligosaccharide is, by acid hydrolysis, released from combination with a phenol, it may be characterized by paper chromatography, paper electrophoresis, and the preparation of suitable derivatives. For an oligosaccharide, where the problem may be a little more difficult, a large number of other methods are available to assist in the determination of structure.[159] There are, in addition, methods used for the examination of the glycosyl residues *in situ*, and special methods, which yield useful structural information, for removing these residues from the aglycons.

Preliminary information regarding the size of the glycosyl substituent may be obtained by paper-chromatographic analysis of the intact phenol–carbohydrate derivative. In solvent systems composed largely of organic components, the greater the number of glycosyl residues, the smaller the R_F value. In highly aqueous systems, the reverse is generally true. Such results, should, however, always be accepted with caution, as differing aglycons may also have pronounced effects on the R_F value. Harborne[156] has reviewed the chromatographic behavior of the flavonoid glycosides. For the anthocyanins, paper chromatography has largely replaced the

(155) E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, **4**, 427 (1950).
(156) J. B. Harborne, *Chromatog. Rev.*, **1**, 223 (1959); **2**, 105 (1960).
(157) Reviewed by L. Hörhammer, in "Methods in Polyphenol Chemistry," J. B. Pridham, ed., Pergamon Press, London (1964), p. 89.
(158) Reviewed by J. B. Pridham, in Ref. 157, p. 111.
(159) Reviewed by R. W. Bailey and J. B. Pridham, *Advan. Carbohydrate Chem.*, **17**, 121 (1962).

determination of distribution numbers (relative partition between two immiscible solvents[160]) used to classify these glycosides. The electrophoretic mobility is inversely related to the size of the glycosyl residue,[161] provided that no complications arise from charged-complex formation between a buffer component and the glycosyl group. A knowledge of the molecular proportions of phenol and monosaccharide liberated by hydrolysis, together with an examination of the substitution pattern of the original phenolic derivative, may also be important to discovering the nature of the glycosyl residue. For example, if a polyphenol glycoside has only one phenolic hydroxyl group substituted with a glycosyl residue, a determination of the aglycon and sugar released by acid hydrolysis provides a direct measure of the size of the glycosyl residue. Phenolic substitution patterns are usually determined by methylation and hydrolysis, or by spectrophotometry. The former is a classical procedure which has been applied widely. The methylating reagents used include diazomethane, which preferentially reacts[94,162] with phenolic hydroxyl groups; methyl iodide and silver oxide, which methylates[115] phenolic and alcoholic hydroxyl groups; and dimethyl sulfate, which methylates both types of hydroxyl group.[102,145] Permethylation may also be achieved with Kuhn's reagent[163] (methyl iodide in *N*,*N*-dimethylformamide) under mild conditions, but only one example of the use of this reagent with phenol-carbohydrate derivatives[164] appears to have been recorded.

The use of spectrophotometry for determining phenolic substitution patterns is more limited in its application, but it has the advantage that only very small amounts of material are required. A review of the spectral properties of flavonoids has been made by Jurd,[165] and of polyphenols in general by Harborne.[166] Of particular interest are studies by Harborne[167] on the spectra of anthocyanins, wherein 3-*O*-glycosyl and 3,5-di-*O*-glycosyl derivatives have been shown to exhibit characteristic absorption maxima. The technique has also been used with phenolic glycosides of lower molecular weight[162] and to distinguish between glucose esters and glucosides of hydroxycinnamic acids.[38]

Most of the remaining procedures used for structural studies are degradative, and result in the release of the combined sugar, which may then

(160) R. Willstätter and E. H. Zollinger, *Ann.*, **412,** 195 (1917). R. Robinson and A. R. Todd, *J. Chem. Soc.*, 2293 (1932).
(161) J. B. Pridham, *Chem. Ind.* (London), 1172 (1961).
(162) J. B. Pridham and M. J. Saltmarsh, *Biochem. J.*, **87,** 218 (1963).
(163) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67,** 32 (1955).
(164) J. B. Pridham and M. J. Saltmarsh, *Biochem. J.*, **82,** 44P (1962).
(165) L. Jurd, in Ref. 5, p. 107.
(166) J. B. Harborne, in Ref. 157, p. 13.
(167) J. B. Harborne, *Biochem. J.*, **70,** 22 (1958).

be identified by the methods of carbohydrate chemistry. Many of these degradative procedures, however, possess elements of specificity, and the fact that a reaction occurs at all may yield valuable information. Periodate oxidation,[167a] a useful tool in the oligosaccharide field, has not found wide application with phenol–carbohydrate derivatives, largely because many phenols are themselves attacked by the reagent. Where the phenolic aglycon is stable to the reagent, however, periodate may be used. Thus, the structure of furcatin (see p. 391) was predicated mainly from the results of oxidation studies on the intact glycoside.

The method most widely used for completely removing the sugar residues from a phenolic glycoside is acid hydrolysis, usually with 1.0–2.0 *N* mineral acid at 100°. The D-glucopyranosiduronic acids are difficult to cleave by this procedure, and require prolonged heating. 7-*O*-Glucosylflavonols and anthocyanidins are also relatively stable to acid,[168] although to a lesser extent than the glycosides of D-glucuronic acid. Purification of aryl glycosides may sometimes be achieved by acetylation, and the acetates may be hydrolyzed directly to the sugar and the aglycon by refluxing with methanolic hydrochloric acid.[169a,169b] Boron trichloride may also be used for the cleavage of *O*-glycosylated cinnamic acid derivatives[170] and flavonoid glycosides,[171] but this reagent also hydrolyzes ether and ester groups from the aglycon. Hydrolysis of glycosylated cinnamic acid derivatives with mineral acid may destroy the cinnamic acid.[44,172]

Hydrolysis on a microscale necessitates identification of the resulting monosaccharides by paper chromatography and paper electrophoresis. These methods are usually reliable with the monosaccharides, provided that a number of different solvent systems are used with the former, and different electrolyte solutions with the latter. An aid to the identification of D-glucose in microgram quantities is the highly specific enzyme D-glucose oxidase, which converts D-glucose into D-gluconic acid and has been used by Harborne[38] for confirming the presence of this aldohexose in hydrolyzates of glycosides. D-Galactose oxidase (D-galactose:O_2 oxidoreductase)[173] and D-galactose dehydrogenase (D-galactose:NAD oxidoreductase)[174] can similarly be used for the identification of D-galactose. Quantitative micro-

(167a) J. M. Bobbitt, *Advan. Carbohydrate Chem.*, **11**, 1 (1956).
(168) J. B. Harborne, *Chem. Ind.* (London), 222 (1962).
(169a) M. K. Seikel, *J. Am. Chem. Soc.*, **72**, 5725 (1950).
(169b) M. K. Seikel, *J. Am. Chem. Soc.*, **77**, 5685 (1955).
(170) E. J. Bourne, N. J. Macleod, and J. B. Pridham, *Phytochemistry*, **2**, 225 (1963).
(171) R. S. Andrews and J. B. Pridham, unpublished results.
(172) R. K. Ibrahim and G. H. N. Towers, *Arch. Biochem. Biophys.*, **87**, 125 (1960).
(173) G. Avigad, D. Amaral, C. A. Sensio, and B. L. Horecker, *J. Biol. Chem.*, **237**, 2736 (1962).
(174) J. C. Su and W. Z. Hassid, *J. Biol. Chem.*, **235**, PC 36 (1960).

analysis may be performed with these enzymes. In addition, in conjunction with paper chromatography, colorimetric methods are available, using, for example, *p*-anisidine hydrochloride[175] or aniline phthalate[176] as spray reagents.

Less drastic treatment with acid may bring about the partial hydrolysis of an aryl glycoside. This is a valuable technique, used extensively for determining the structure of oligoglycosyl residues. Wender and coworkers[177] have, for example, used formic acid in cyclohexanol for hydrolyzing a number of *O*-rhamnosylglucosyl derivatives of flavonoids to the corresponding glucosides. The rutinoside naringin (4′,5-dihydroxy-7-rutinosyloxyflavone) has also been degraded to the corresponding 7-β-D-glucoside by refluxing a solution of it in methanol and hydrochloric acid.[169b] Hydrochloric acid has been used for the stepwise hydrolysis of oligosaccharide glycosides of anthocyanidins,[152,178–180] and rhamnose has been preferentially removed from 4′-*O*-arabinosyl-3-*O*-rhamnosylkaempferol by treating it with formic acid[104] at 75°.

Oligosaccharides in combination with phenols may also be removed intact by careful hydrolysis with acid. Thus, when rutin is heated with 10% aqueous acetic acid, it yields rutinose.[38] Dilute mineral acids can, for example, be used for the removal of sophorose from kaempferol 3-sophoroside,[107] and of gentiobiose from *p*-hydroxyphenyl β-gentiobioside.[181] A comparison of the use of formic acid–cyclohexanol and hydrochloric acid–acetic acid for the partial hydrolysis of glycosides has been made by Chandler and Harper.[182] There appears to be no general rule governing the use of these acids.

Such esters of sugars as β-D-glucopyranosyl caffeate, in addition to being cleaved by acid, are saponified by dilute alkali under mild conditions, with the liberation of the free reducing sugar. Alkali is a useful reagent for distinguishing between the glucosides of hydroxycinnamic acids and its glucose esters.[38] The former are relatively stable to alkali, although oxidative degradation may occur with both types of derivative; hence, alkaline hydrolyses are best performed under nitrogen.

Hydrolyses, both partial and complete, may be effected with enzymes. The advantages of using these catalysts are (a) that the conditions of

(175) J. B. Pridham, *Anal. Chem.*, **28,** 1967 (1956).
(176) C. M. Wilson, *Anal. Chem.*, **31,** 1199 (1959).
(177) D. W. Fox, W. L. Savage, and S. H. Wender, *J. Am. Chem. Soc.*, **75,** 2504 (1953).
(178) R. Willstätter and E. K. Bolton, *Ann.*, **412,** 113 (1916).
(179) Y. Abe and K. Hayashi, *Botan. Mag.* (Tokyo), **69,** 577 (1956); *Chem. Abstracts*, **52,** 9327 (1958).
(180) J. B. Harborne, *Biochem. J.*, **74,** 262 (1960).
(181) J. D. Anderson, L. Hough, and J. B. Pridham, *Biochem. J.*, **77,** 564 (1960).
(182) B. V Chandler and K. A. Harper, *Australian J. Chem.*, **14,** 586 (1961).

reaction are mild, and (b) information regarding the nature of the glycosidic linkage (and, sometimes, other configurational details) may be obtained. Where only small quantities of the material are available, enzymes must be wholly relied on to reveal the anomeric configuration. Other methods for this purpose include measurements of optical rotation and infrared spectra, both of which, in comparison with the enzymic methods, require relatively large amounts of the compound. The absorption bands said to be characteristic of α-D and β-D links[183] are rather weak, and several milligrams are required for infrared analysis. Enzymic hydrolysis may be performed on the microgram scale. The failure of an enzyme to react with an aryl glycoside may be due to an absence of the necessary specific carbohydrate residue; however, in some cases, this may be present, but hydrolysis is hindered by the aglycon. The latter is an important point which should be borne in mind.

The enzyme preparation most widely used is almond emulsin, or a purified β-D-glucosidase fraction from almond or a related plant. Emulsin exhibits a complex of hydrolase activities,[184] three of the major functions being as a β-D-glucosidase and as an α- and a β-D-galactosidase. In view of this mixed activity, preparations should be used with caution, although there is little doubt that emulsin accurately distinguishes between an α- and a β-D-glucopyranoside in most instances. 3-*O*-β-D-Glucosyl derivatives of anthocyanidins, however, are not hydrolyzed by almond emulsin. Hydrolysis of these compounds requires an anthocyanase preparation obtained from *Aspergillus niger*.[185] This preparation is a complex mixture of enzymes; the specificity has been examined by Harborne and Sheratt.[186] It hydrolyzes maltose and methyl α-D-glucopyranoside. It may be conjectured that it would hydrolyze anthocyanidin α-D-glucosides, but the preparation has not yet been tested with these compounds. Anthocyanase has also been used as a source of esterase for the hydrolysis of glucose cinnamates.[38] Mouse-liver β-D-glucuronidase, as already mentioned (see p. 384), hydrolyzes flavonoid β-D-glucopyranosiduronic acids,[85,87] and a yeast glycosidase preparation has been used for the hydrolysis of polystachoside, a β-L-arabinoside.[101] An *Aspergillus oryzae* preparation selectively removes the rhamnosyl residue from 4′-*O*-arabinosyl-3-*O*-rhamnosylkaempferol.[104]

Various species of *Rhamnus* provide an enzyme mixture, commonly

(183) S. A. Barker, E. J. Bourne, and D. H. Whiffen, *Methods Biochem. Anal.*, **3**, 213 (1956).

(184) Reviewed by J. B. Pridham, in "Enzyme Chemistry of Phenolic Compounds," J. B. Pridham, ed., Pergamon Press, London, 1963, p. 73.

(185) H. T. Huang, *J. Am. Chem. Soc.*, **78**, 2390 (1956).

(186) J. B. Harborne and H. S. A. Sherratt, *Biochem. J.*, **65**, 24P (1957).

called rhamnodiastase. These enzyme systems, used extensively by French workers in the early part of this century, are valuable for studies of aryl glycosides, as they possess so-called disaccharidase activity. With many disaccharide glycosides, the glycosidic linkage adjacent to the phenolic aglycon is preferentially hydrolyzed. For example, rutin is hydrolyzed to quercetin and rutinose,[187] and robinin to 7-*O*-rhamnosylkaempferol and robinobiose.[188] Chandler and Harper[182] have shown that an enzyme preparation from *Rhamnus frangula* hydrolyzes various flavonoid 3-mono- and di-glycosides (including anthocyanins). Suzuki[92] studied in some detail a preparation from *Rhamnus dahurica*, and showed that an α-L-rhamnosidase and two different β-D-glucosidases were present. This mixture of enzymes also hydrolyzed anthocyanidin glycosides. Thus, 3-*O*-rutinosylcyanidin yielded rutinose, together with some glucose and rhamnose; and such compounds as cyanin and chrysanthemin, having monoglucosyl groups, were also hydrolyzed. The flavanone glycosides, naringin and hesperidin (3′,5-dihydroxy-4′-methoxy-7-rutinosyloxyflavanone), were not cleaved by the preparation from *Rhamnus*, but some flavonol glycosides, such as 3-*O*-glucosylquercetin, were. Imaseki and Yamamoto[189] have detected in *Viburnum furcatum* a disaccharidase which liberates 6-*O*-D-apiofuranosyl-D-glucopyranose from furcatin. This enzyme has no action on the related glycoside apiin, which possesses a (1→2)-linked apiosyl-glucosyl substituent, and also will not hydrolyze rutin.

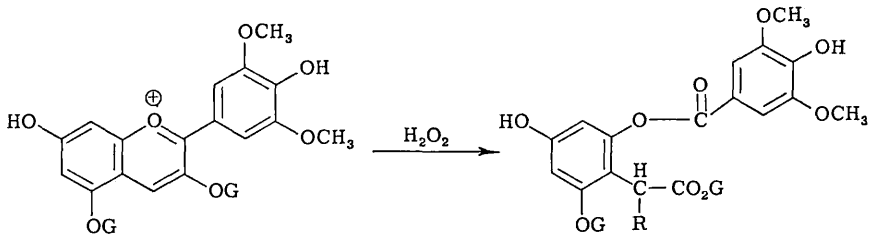
p-Hydroxyphenyl glucosides readily undergo oxidative cleavage with dilute bromine water[150,171,190] or ferric chloride solution.[191] Under these mild conditions, benzoquinone is formed, and the mono- or oligo-glucosyl residues are liberated as reducing sugars. Similar results have been obtained by using an oxidase from potato.[150,189,192] Presumably, other compounds possessing potential quinonoid hydroxyl groups substituted with glycosyl residues would also undergo this facile oxidative cleavage. This reaction would be valuable for releasing oligosaccharides, in relatively high yields, from combination with phenols.

Potassium permanganate has been used for the controlled, oxidative rupture of the phenolic nuclei from flavonoid glycosides, with little concomitant degradation of the carbohydrates liberated.[182] This procedure permits identification of all of the glycosyl residues present in a flavonoid, provided that the flavonoid can be oxidized. Highly methylated deriva-

(187) C. Charaux, *Bull. Soc. Chim. Biol.*, **6**, 631 (1924); G. Zemplén and R. Bognár, *Ber.*, **75**, 482 (1942).
(188) G. Zemplén and A. Gerecs, *Ber.*, **68**, 2054 (1935).
(189) H. Imaseki and T. Yamamoto, *Arch. Biochem. Biophys.*, **92**, 467 (1961).
(190) C. E. Cardini, *Ciencia Invest.* (Buenos Aires), **17**, 349 (1961).
(191) J. B. Pridham and M. J. Saltmarsh, unpublished results.
(192) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937).

tives, for example, might be stable to oxidative attack. Ozonolysis, followed by hydrolysis, may also be used to rupture the flavonoid nucleus,[193] and this technique has been developed for use on a microscale by Chandler and Harper.[182]

The controlled oxidation of anthocyanins with hydrogen peroxide was investigated by Karrer and de Meuron.[194] They showed that malvin (**22**) can be converted into malvone (**23**) as follows. In malvone (**23**), the



(22) (23)

(R = H or OH, not known)

glucose residue attached to the hydroxyl group at C-3 is also attached, by an ester linkage, to the phenol, and, consequently, Karrer and de Meuron[194] were able to remove it by alkaline hydrolysis. The glucosyl residue attached at C-5 was removed at a later stage with acid. Similar results were obtained with hirsutin (3,5-di-D-glucosyloxy-4′-hydroxy-3′,5′,7-trimethoxyflavylium). Chandler and Harper[182] showed that this method is good for identifying mono- and di-saccharides bonded glycosidically to the hydroxyl group at C-3 of flavonols and anthocyanidins.

IV. Biosynthesis

A great deal of information regarding the biosynthesis of phenol–carbohydrate derivatives has been obtained by feeding phenols to plants or detached plant organs. The earliest record of such work is a publication in 1916 by Ciamician and Ravenna,[195] who claimed that when such simple phenols as quinol and saligenin (*o*-hydroxybenzyl alcohol) are fed to maize seedlings, they are converted into the corresponding aryl D-glucosides. No similar experiments with phenols were reported until 1940, when

(193) L. Mester, *Magy. Kem. Folyoirat*, **50**, 125 (1944); *Chem. Abstracts*, **46**, 8018 (1952).
(194) P. Karrer and G. de Meuron, *Helv. Chim. Acta*, **15**, 507, 1212 (1932).
(195) G. Ciamician and C. Ravenna, *Atti Reale Accad. Lincei*, **25**, 3 (1916).

Miller[196] treated gladiolus corms with *o*-chlorophenol, and isolated relatively large quantities of *o*-chlorophenyl β-gentiobioside. Miwa and coworkers[197] infused leaf discs with various phenols together with D-glucose, and showed that mono-β-D-glucosides were produced. When the D-glucose was replaced by α-D-glucosyl phosphate, increased yields of the aryl β-D-glucosides resulted. Grinding of the tissues was shown to decrease the synthetic activity, but, from *Pinus sativa* and *Vicia faba* seeds, cell-free enzyme preparations synthesized the β-D-glucosides, provided that adenosine 5-triphosphate and an extract of yeast were also present. Other feeding experiments with leaves[198] and with germinating seeds[162] confirmed much of the Japanese work. For *Vicia faba*,[162] the glycosylation appeared to be relatively nonspecific; with trihydric phenols, for example, all of the possible, isomeric, mono-D-glucosyl derivatives were formed. Nystrom, Tolbert, and Wender[199] have demonstrated that when phenol is fed, in low concentration, to barley or wheat leaves, it is converted into phenyl β-D-glucopyranoside. When germinating *Vicia faba* seeds are fed with resorcinol, *m*-hydroxyphenyl β-D-glucoside is formed, together with a compound tentatively identified as a sulfated form of this D-glucoside.[164] Pridham[200] has surveyed a large number of different plant species, and has shown that the ability to D-glucosylate foreign phenols is probably common to all angiosperms and gymnosperms, and, perhaps, to ferns also. Only some species of moss, and no algae, appear to be capable of this reaction.[201]

When hydroxycinnamic acid derivatives are fed to plants, the situation is more complicated, in that both aryl D-glucosides and esters of D-glucose may be produced. Thus, Kosuge and Conn[202] have shown that *o*-coumaryl β-D-glucoside (2-β-D-glucosyloxy-*trans*-cinnamic acid) is formed by leaves of sweet clover which have been treated with *o*-coumaric acid (2-hydroxycinnamic acid). Harborne and Corner,[38] however, with a number of different plant species, could only demonstrate the formation of a D-glucose ester after a short treatment with *o*-coumaric acid. After several days, a di-D-glucosyl ester derivative was formed, probably an (*o*-hydroxycinnamoyl)gentiobiose. Harborne and Corner[38] also showed that D-glucose esters are the main products formed with a number of other hydroxy- and methoxy-cinnamic acids. The only exception to the rule was caffeic

(196) L. P. Miller, *Science*, **92**, 42 (1940); *Contrib. Boyce Thompson Inst.*, **12**, 163 (1941).
(197) T. Miwa, S. Nakamura, and A. Shibata, *Koso Kagaku Shimpoziumu*, **12**, 48 (1957); *Chem. Abstracts*, **52**, 1314 (1958).
(198) A. Hutchinson, C. Roy, and G. H. N. Towers, *Nature*, **181**, 841 (1958); J. B. Pridham, *ibid.*, **182**, 795 (1958).
(199) C. W. Nystrom, N. E. Tolbert, and S. H. Wender, *Plant Physiol.*, **34**, 142 (1959).
(200) J. B. Pridham, *Phytochemistry*, **3**, 493 (1964).
(201) C. Roy, M. Sc. Thesis, McGill University, Montreal, 1959.
(200) J. B. Pridham, unpublished results.

acid, which, when fed to *Lycopersicum esculentum* leaves, was partially converted into 3- and 4-*O*-β-D-glucosylcaffeic acids. Runeckles and Woolrich[203] demonstrated the formation both of D-glucose esters and aryl D-glucosides when *p*-coumaric acid and ferulic acid were fed to tobacco leaf discs. Further work is required before the significance of the above three studies becomes comprehensible. The results, as they stand, may be due to a species difference or to the failure to detect all of the D-glucosylated products that are formed.

The first clue regarding the detailed mechanism of the formation of aryl glycosides came from studies of Miwa and coworkers[197] which suggested that nucleotide cofactors are necessary. It was left to Cardini and Leloir,[204] however, to show that the nucleotide probably directly involved is uridine 5-(α-D-glucopyranosyl pyrophosphate) (**24**). These workers incubated quinol with compound (**24**) and a wheat-germ extract, and by paper chromatography detected arbutin, a mono-D-glucoside of quinol, which was formed. This observation was later substantiated by Pridham and Saltmarsh[162,205] using a crude, enzyme preparation from *Vicia faba* seeds and a number of different mono-, di-, and tri-hydric phenols. The products obtained from these experiments were similar to those formed when the same phenols are introduced into germinating bean seeds. The transfer of D-glucose *in vitro* did, however, appear to be more specific, in that, with the polyhydric aromatic compounds, only one of the possible isomeric mono-D-glucosides is formed, and this corresponds to the major component produced in the feeding experiments. For example, feeding of pyrogallol produced the 2,3- and 2,6-dihydroxyphenyl β-D-glucosides in the approximate ratio of 3:1. *In vitro* in the presence of (**24**), only the former compound was synthesized. Mono-β-D-glucosides of dihydric phenols are also synthesized *in vitro* by utilizing the uridine 5-triphosphate (**25**):α-D-glucosyl phosphate uridylyltransferase, adenosine 5-triphosphate (**26**):(**25**) phosphotransferase and (**24**):phenol D-glucosyltransferase activities of a wheat-germ extract, thus:

$$UP_3\ (\mathbf{25}) + \alpha\text{-D-G-1-P} \rightarrow UP_2\text{-D-G} + \text{pyrophosphate}$$

$$UP_2\text{-D-G} + ArOH \rightarrow ArO\text{-D-G} + UP_2$$

$$AdoP_3\ (\mathbf{26}) + UP_2 \rightarrow UP_3 + AdoP_2$$

where UP_3 is uridine 5-triphosphate, UP_2 is uridine 5-pyrophosphate,

(201) C. Roy, M. Sc. Thesis, McGill University, Montreal, 1959.
(202) T. Kosuge and E. E. Conn, *J. Biol. Chem.*, **234**, 2133 (1959).
(203) V. C. Runeckles and K. Woolrich, *Phytochemistry*, **2**, 1 (1963).
(204) C. E. Cardini and L. F. Leloir, *Ciencia Invest.* (Buenos Aires), **13**, 514 (1957).
(205) J. B. Pridham and M. J. Saltmarsh, *Biochem. J.*, **74**, 42P (1960).

D-G is D-glucose, Ar is aryl, $AdoP_3$ is adenosine 5-triphosphate, and $AdoP_2$ is adenosine 5-pyrophosphate. Yamaha and Cardini[206] have made a detailed study of the UP_2G:phenol D-glucosyltransferase activity present in wheat germ. Crude extracts D-glucosylated phenols having various arrangements of hydroxyl groups, but fractionation of the extract with ammonium sulfate and alumina gel yielded a preparation which was fairly specific for the mono-D-glucosylation of phenols having two hydroxyl groups *para* to each other. This study suggested that in plant tissues several such enzymes may be present, each being specific for a particular configuration of phenolic hydroxyl groups. Attempts to D-glucosylate phenolic hydroxyl groups by replacing (**24**) by α-D-glucosyl phosphate and relatively simple α- and β-D-glucosides failed for both the broad-bean and wheat-germ enzyme preparations.

Other reports of transfer of D-glucose from (**24**) to phenolic hydroxyl groups have been made by Dutton and Duncan,[207] using a sugar-beet homogenate and *o*-aminophenol as an acceptor, and by Barber[208] with a mung-bean enzyme preparation and quercetin. In the latter experiment, the product of the reaction was an *O*-β-D-glucosyl derivative, substitution probably having occurred at the hydroxyl at C-3 of the flavonol; thymidine 5-(D-glucosyl pyrophosphate) (**27**) also serves as a D-glucose donor in this system. Adenosine 5-(D-glucosyl pyrophosphate) (**28**) is reported to be a better donor of D-glucose than (**24**) in the presence of quinol and a wheat-germ enzyme.[209] Under these same conditions, guanosine 5-(D-glucosyl pyrophosphate) and cytidine 5-(D-glucosyl pyrophosphate) act as weak donors of D-glucose.

In two instances, the enzymic transfer of D-glucose from (**24**) to carboxylic acid groups has been demonstrated by using plant preparations,[202,210] and it is probable that the biosynthesis of all of the D-glucose cinnamates also occurs by this mechanism.

The formation of an aryl di-D-glucoside *in vitro* was first observed when arbutin was converted into *p*-hydroxyphenyl β-gentiobioside by β-D-glucosidase preparations from aspen cambium, almond seeds, and broad-bean seeds.[175,181] It was assumed that the arbutin acts as both a donor and an acceptor, and that the β-D-glucosidase transfers D-glucosyl residues to the primary hydroxyl groups of the arbutin molecule. Cardini and Yamaha[211] reported that wheat-germ extracts, possessing no β-D-glucosidase

(206) T. Yamaha and C. E. Cardini, *Arch. Biochem. Biophys.*, **86,** 127 (1960).
(207) G. J. Dutton and A. M. Duncan *Biochem. J.*, **77,** 18P (1960).
(208) G. A. Barber, *Biochemistry*, **1,** 463 (1962).
(209) J. C. Trivelloni, E. Recondo, and C. E. Cardini, *Nature*, **195,** 1202 (1962).
(210) G. Jacobelli, M. J. Tabone, and D. Tabone, *Bull. Soc. Chim. Biol.*, **40,** 955 (1958).
(211) C. E. Cardini and T. Yamaha, *Nature*, **182,** 1446 (1958).

activity, catalyze the transfer of D-glucose from (**24**) to arbutin, with the formation of *p*-hydroxyphenyl β-gentiobioside. The extract was later fractionated, and an enzyme specific for the further D-glucosylation of aryl β-D-glucosides was isolated.[212] The highest rates of reaction were obtained when phenyl β-D-glucopyranoside, salicin, and arbutin were used as acceptors. *m*-Methoxyphenyl and *m*-hydroxyphenyl β-D-glucopyranoside were only D-glucosylated to the extent of 51% and 33%, respectively, of the rate of arbutin. No reaction was observed with free phenols. Compound (**28**) also functions as a D-glucose donor for synthesis of β-gentiobioside.[209] A triglucoside possessing two β-D-(1→6) linkages is also formed when quinol is incubated with (**24**) and a wheat-germ extract.[150]

The enzymic transfer of monosaccharides other than D-glucose from nucleotide derivatives to phenolic compounds has also been observed for a *Phaseolus vulgaris* enzyme which catalyzes the formation of a β-D-glucosiduronic acid of quercetin from uridine 5-(D-glucosyluronic acid pyrophosphate) (**29**) and the flavonol. The position of the D-glucosiduronic acid residue is not yet known.[213] L-Rhamnose can also be transferred from thymidine 5-(L-rhamnosyl pyrophosphate) (**30**) to 3-*O*-β-D-glucosylquercetin, with the formation of a product that appears to be the corresponding β-rutinoside.[210] The enzyme preparation was, in this instance, obtained from *Phaseolus vulgaris* leaves. *In vivo*, the L-rhamnose donor is more likely to be uridine 5-(L-rhamnosyl pyrophosphate) (**31**).[214]

The following conclusions regarding the mechanism of formation of aryl glycosides *in vivo* may now be drawn. In most cases, it is probable that the first glycosyl residue is transferred to the phenol at a late stage of biosynthesis; with a flavonoid, this would be after the $C_6:C_3:C_6$ nucleus has been formed.[215] It has, however, been suggested, from purely chemical studies, that near-precursors of some phenols might be glycosylated prior to the completion of the phenol biosynthesis.[216] There is as yet little biochemical evidence to support this theory.

The biosynthesis of salicin, a β-D-glucoside of low molecular weight, probably does not occur by glycosylation of saligenin, as all attempts to produce salicin from saligenin *in vivo* and *in vitro* have afforded the isomeric *o*-hydroxybenzyl β-D-glucopyranoside as the main product.[185] The precursor of salicin might well be the β-D-glucoside of salicylaldehyde.[162,216a] With compounds having complex glycosyl residues, it is almost certain

(212) C. E. Cardini and T. Yamaha, *Arch. Biochem. Biophys.*, **86**, 133 (1960).
(213) C. A. Marsh, *Biochim. Biophys. Acta*, **44**, 359 (1960).
(214) G. A. Barber, *Biochem. Biophys. Res. Commun.*, **8**, 204 (1962).
(215) T. A. Geissman and E. Hinreiner, *Botan. Rev.*, **18**, 77 (1952).
(216) L. Reichel and R. Schickle, *Ann.*, **553**, 98 (1942).
(216a) J. B. Pridham and M. Young, *Biochem. J.*, **92**, 20P (1964).

that residues are added stepwise, and not as intact units. Much support for this theory is derived from studies of the co-occurrence of related glycosides within a species.[71,112,154,180]

As regards the actual transfer of glycosyl residues to phenolic hydroxyl groups, a number of different enzymes probably catalyze these reactions, and some specificity is shown toward the donor and the acceptor molecules. Possibly, several enzymes exist which catalyze the transfer of glycosyl units to existing glycosyl substituents, and these would appear to be distinct from those enzymes catalyzing transfer to phenolic hydroxyl groups.

Strong circumstantial evidence has been educed suggesting that the D-glucose donor molecule for synthesis of D-glucosides in plant tissues is a nucleoside pyrophosphate derivative of D-glucose, probably (**24**). Points in favor of this are: (*i*) (**24**) functions as a donor *in vitro*, and (*ii*) a reaction involving (**24**) would be energetically favorable in comparison with transfer of D-glucose from a low-energy source. The latter type of reaction has, so far, not been demonstrated with enzyme systems of higher plants. (*iii*) Compound (**24**) is probably present in all higher plants, and details of its formation in plants from α-D-glucosyl phosphate and (**25**):α-D-glucosyl phosphate uridylyltransferase, as well as by other pathways, are well known.[217,218] The transfer of groups other than D-glucosyl presumably involves the corresponding nucleoside pyrophosphate derivatives. Often, this is probably a uridine derivative, although other nucleosides may well participate in these reactions. Nucleoside pyrophosphate derivatives of all the monosaccharides, except D-apiose, which occur in combination with phenols are known[218] to occur in Nature. Probably, all of the D-monosaccharides in combination with nucleoside pyrophosphates have the α-D anomeric configuration, and these compounds may be formed from the corresponding α-D-glycosyl phosphates in the presence of specific nucleoside 5-triphosphate:α-D-glycosyl phosphate uridylyltransferases. As would be expected, the β-L-glycosyl phosphates of L-monosaccharides are converted into nucleoside pyrophosphate derivatives, and these presumably possess β-L glycosidic linkages.[218] However, any chemical evidence for the fine structures of most of these sugar nucleotides, with the exception of (**24**), is exceedingly sparse.

Accepting the above structures, the transfer of monosaccharide residues from nucleoside pyrophosphate derivatives to phenolic hydroxyl groups appears to result in inversion of the anomeric configuration of the glycosyl residue.[162,206,208,209,213] This is most certainly true of the formation of aryl

(217) W. Z. Hassid, E. F. Neufeld, and D. S. Feingold, *Proc. Natl. Acad. Sci. U. S.*, **45**, 905 (1959).

(218) W. Z. Hassid, *Biochem. Soc. Symp.* (Cambridge, Engl.), **21**, 63 (1961). E. F. Neufeld and W. Z. Hassid, *Advan. Carbohydrate Chem.*, **18**, 309 (1963).

β-D-glucosides. Similarly, inversion occurs on transfer of D-glucosyl residues from (24)[212] and (28)[209] to the D-glucosyl moiety of arbutin, and probably also occurs when L-rhamnosyl residues are transferred from (30)[208] or (31)[214] to 3-*O*-β-D-glucosylquercetin. The formation of the various quercetin 3-L-arabinosides reported to occur in Nature[100–102] is difficult to explain on the basis of this general mechanism. In addition, a donor molecule for D-apiose has yet to be found.

Although several attempts to synthesize α- and β-D-glucosyl derivatives of phenols failed when enzyme systems of higher plants were used in the absence of sugar nucleotides,[162,181,211,219] enzymes from mycelium of *Aspergillus niger* transfer D-glucose from maltose (and isomaltose) to resorcinol, with the formation of *m*-hydroxyphenyl α-D-glucopyranoside and two higher derivatives, probably the corresponding isomaltoside and maltoside.[161] Also, the β-D-galactosidase of *Escherichia coli* produces *m*-hydroxyphenyl β-D-galactopyranoside by the transfer of D-galactose from lactose to resorcinol.[220] There is, however, no evidence that this type of reaction occurs *in vivo* in fungi and bacteria. Nakamura and Miwa[220a] have utilized the energy of the glycosidic bond in sucrose, and have achieved mono-D-fructosylation of quinol in the presence of invertase (β-D-fructofuranoside fructohydrolase) preparations from micro-organisms and wheat.

V. Metabolism and Function

In Section II of this article, some indication was given of the many different types of phenol–carbohydrate derivatives which occur in plants. Within a single plant species, there may be a great diversity of structure, and one phenol may exist as several glycosides. For example, some apple skins contain six different quercetin glycosides,[99] and *Vaccinium myrtillus* leaves contain five.[221] This discovery immediately posed the question as to the function such a mixture plays in the life of the plant; the answer is still unknown. The classical ideas regarding the function of glycosides have been reviewed by Pridham.[222] Stabilization, solubilization, and detoxification of phenols may be valid reasons for glycoside formation, and phenol–carbohydrate derivatives no doubt play some role in disease resistance. It was originally believed that aryl glycosides constitute sugar reserves, but it is doubtful that they are important reserves in healthy

(219) J. B. Pridham, *Biochem. J.*, **76,** 13 (1960).
(220) J. B. Pridham and K. Wallenfels, *Nature*, **202,** 488 (1964).
(220a) S. Nakamura and T. Miwa, *Nature*, **202,** 91 (1964).
(221) C. H. Ice and S. H. Wender, *J. Am. Chem. Soc.*, **75,** 50 (1953).
(222) J. B. Pridham, in "Phenolics in Plants in Health and Disease," J. B. Pridham, ed., Pergamon Press Oxford, 1960, p. 9.

plants. Other workers, for example, Straub,[223] took the opposite view, and considered that aryl glycosides are waste products not further metabolized. It now seems probable that some D-glucosides are important metabolites and are involved in the biosynthesis of lignin[224] and coumarin[225]; they may also be concerned in the control of 3-indolylacetic acid oxidase activity in plant tissues.[226]

In connection with the more-complex glycosyl residues which are bonded to phenols, it is tempting to suggest that these represent the primer molecules for the biosynthesis of polysaccharides (heteroglycans). Such primers might first be elaborated on a phenolic nucleus; then the phenol might be removed by enzymic hydrolysis or oxidation before further elongation of the monosaccharide chains occurred, or the phenol might remain in place during chain elongation. At present, however, no structural relationship between polysaccharides and the complex glycosidic residues known has been reported, and there is no real evidence to support the hypothesis.

(223) W. Straub, *Biochem. Z.*, **82,** 48 (1917).
(224) Reviewed by K. Freudenberg, *Pure Appl. Chem.*, **5,** 9 (1962).
(225) T. Kosuge and E. E. Conn, *J. Biol. Chem.*, **236,** 1617 (1961).
(226) M. Furaya, A. W. Galston, and B. B. Stowe, *Nature*, **193,** 456 (1962).
(227) M. Piattelli, L. Minale, and G. Prota, *Ann. Chim.* (Rome), **54,** 955 (1964). M. E. Wilcox, H. Wyler, T. J. Mabry, and A. S. Dreiding, *Helv. Chim. Acta*, **48,** 252 (1965).

Addendum

Structure (**14**) for betanin (see p. 375) has been shown to be slightly incorrect.[227]

B18

(*Reprinted from Nature, Vol. 205, No. 4977, pp. 1213–1214, March 20, 1965*)

Structure of a Dopa Glucoside from *Vicia faba*

β-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) has only been detected in a limited number of plants, and it appears to be confined mainly to leguminous species. Unlike most other phenols of low molecular weight, however, it exists in relatively high concentrations in the free state. This is particularly the case, for example, with the broad-bean, *Vicia faba*[1], but Nagasawa and his associates[2] have also isolated an *O*-β-D-glucoside of DOPA from the testa of the bean seed. The Japanese workers did not, however, determine whether it was a 3- or 4-*O* substituted derivative and in view of our interest in the formation and metabolism of DOPA in plants we have reinvestigated the structure and distribution of this compound.

As *V. faba* contains only small amounts of this DOPA glucoside a preliminary examination of the glucoside obtained in high yield by feeding *Pisum sativum* seeds with DOPA was undertaken and a comparison then made of this compound with the corresponding derivative obtained from the bean.

Seeds of *P. sativum* var. 'Early Onward' were soaked overnight in a saturated solution of L-DOPA. They were then washed and left on moist cotton wool for 4 days in the dark (*cf.* Pridham and Saltmarsh[3]). The resulting seedlings were extracted with aqueous ethanol (80 per cent) and the concentrated extract was examined on paper chromatograms. This showed the presence of at least five compounds which are not normally present in the tissues. By careful fractionation of the extract on paper, using ethyl acetate/acetic acid/water (9 : 2 : 2 v/v) solvent followed by electrophoresis (0·1-*M* formic acid, *p*H 2·4, 4,000 V/1 h), a small quantity of a compound, P_2, was isolated as a syrup which was freeze-dried to a hygroscopic powder. Hydrolysis of P_2 with N H_2SO_4, N HCl, 50 per cent formic acid or β-D-glucoside glucohydrolase yielded only DOPA and D-glucose in the molar ratio of 1 : 1. The configuration of the glucose was determined by reaction with D-glucose oxido-reductase, and the rate of acid hydrolysis of P_2 suggested that the glucosyl residue was in the pyranoid form.

β - (3 - Hydroxy - 4 - methoxyphenyl) - DL - alanine and β-(3-methoxy-4-hydroxyphenyl)-DL-alanine were synthesized as model compounds and their colour reactions with diazotized *p*-nitroaniline/NaOH and ultra-violet spectra under neutral and alkaline conditions determined. This revealed several significant differences between the 3-*O*- and 4-*O*-substituted compounds and in particular a variation in the relative absorbance at ~ 240 and ~ 295 mμ

on addition of alkali. P_2 had all the characteristics of a 3-*O*-substituted DOPA. The absence of a free *O*-dihydroxyl grouping was further indicated by the fact that it gave no colour reaction with aqueous sodium molybdate and did not exhibit a characteristic hypsochromic shift in spectrum under alkaline conditions when treated with borate.

Confirmation of the position of the D-glucopyranosyl residue on the aromatic ring was obtained after methylation of P_2 by the Kuhn procedure with methyl iodide and silver oxide in dimethylformamide[4]. Alkaline nitrobenzene oxidation[5] of the methylated derivative gave rise to isovanillin as the only aldehydic product. This was characterized by paper electrophoresis using phosphate buffers at *p*H 8·7 and 10·0, with 2,4-dinitrophenylhydrazine/NaOH as the locating reagent (isovanillin is readily distinguished from vanillin by this method as the latter possesses a more strongly dissociated phenolic hydroxyl group).

The cotyledons from 4-day-old broad-bean (var. 'Johnson's Longpod') seedlings were extracted and examined by the same procedure, and a compound identical to P_2 in all respects was isolated. The conclusion is, therefore, that both P_2 and the derivative from *V. faba* are β-[3-(β-D-glucopyranosyloxy)-4-hydroxyphenyl]-L-alanine. The colour reactions and chromatographic and electrophoretic behaviour of this compound are summarized in Table 1.

Table 1. PROPERTIES OF DOPA GLUCOSIDE

	P_2 and *V. faba* glucoside
Colour reaction with:	
Diazotized *p*-nitroaniline/NaOH	Plum red—grey green
Ninhydrin	Blue-violet
Sodium molybdate	No reaction
R_{DOPA} *values*	
Butan-1-ol/acetic acid/water (6 : 1 : 2, v/v)	0·47
Butan-1-ol/ethanol/water (40 : 11 : 19, v/v)	0·60
Ethyl acetate/acetic acid/water (9 : 2 : 2, v/v)	0·35
Ethyl acetate/acetic acid/formic acid/water (18 : 3 : 1 : 4, v/v)	0·37
Ethyl acetate/pyridine/water (10 : 4 : 3, v/v)	0·56
Butan-1-ol saturated with water	0·30
M_{DOPA} *values**	
0·1 *M* formic acid (*p*H 2·4)	0·85

* Electrophoretic mobility relative to L-DOPA, corrected for electroendosmosis.

Nagasawa and his colleagues[2] reported that DOPA glucoside occurred mainly in the green testa of a late ripening variety of *V. faba* and to a lesser extent in the hilum and the inner tissue of the pod. Our own chromatographic investigations with the 'Johnson's Longpod' variety confirm their findings but also suggest that this compound is present in both the dormant and germinated cotyledons. There is no evidence, however, for its occur-

rence in either the roots or shoots of young plants or in other issues of the mature plant other than the pods.

Investigations are continuing on the DOPA–DOPA glucoside equilibrium in *V. faba*. The apparent lack of glucoside in the tissues could be due to a weakly active specific glucosylating enzyme (although other phenols are readily glucosylated[3]) or to a physical barrier between the enzyme and its substrate, L-DOPA.

We thank the Agricultural Research Council and the United States Department of Agriculture for financial assistance.

R. S. ANDREWS
J. B. PRIDHAM*

Department of Chemistry,
Royal Holloway College,
University of London,
Englefield Green, Surrey.

* Present address: Department of Biochemistry, University of California, Berkeley 4, California.

[1] Guggenheim, M., *Z. Physiol. Chem.*, **88**, 276 (1913).
[2] Nagasawa, T., Takagi, H., Kawakami, K., Suzuki, T., and Sahashi, Y., *Agr. Biol. Chem.*, **25**, 441 (1961).
[3] Pridham, J. B., and Saltmarsh, M. J., *Biochem. J.*, **87**, 218 (1963).
[4] Kuhn, R., Trischmann, H., and Low, I., *Angew. Chem.*, **67**, 32 (1955).
[5] Stone, J. E., and Blundell, M. J., *Anal. Chem.*, **23**, 771 (1951).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

B 19

Reprinted from
ANNUAL REVIEW OF PLANT PHYSIOLOGY
Vol. 16, 1965

LOW MOLECULAR WEIGHT PHENOLS IN HIGHER PLANTS[1,2]

By J. B. Pridham
Royal Holloway College (University of London), Englefield Green, Surrey, England

Introduction

Phenolic compounds are second only to carbohydrates in abundance in higher plants. They display a great variety of structures ranging from derivatives of simple phenols, such as quinol, to complex polymeric materials, such as lignin.

In recent years, naturally occurring phenolics have been the subject of several reviews. Past editions of this publication alone have contained articles on lignin (1), the biosynthesis of flavonoids (2), and general biosynthetic pathways (3). In other periodicals, accounts of the chemistry and biochemistry of coumarins (4) and lignin (5, 6, 7) can be found.

For the purposes of this review the author intends, wherever possible, to limit the discussion to those naturally occurring compounds which possess only one aromatic ring and to concentrate on the biochemical and physiological aspects which have not received detailed attention in the past.

The compounds which come within these terms of reference and which will receive attention include simple mono-, di-, and trihydroxybenzenes, phenolic alcohols, aldehydes and carboxylic acids, hydroxycinnamic acids, and phenolic amino acids and amines.

Glycosidic Derivatives

Most plant tissues contain complex mixtures of phenols, and with the exception of many wood constituents, the majority are present as glycosidic derivatives. The cell vacuole is generally assumed to be the main storage organ for these glycosides; whether phenols or phenolic glycosides undergo any significant translocation appears to be in dispute. Miller (8), for example, believed that glycosides accumulate at sites of low metabolic activity and remain there until the death of the plant. Gladiolus corms which had been treated with *o*-chlorophenol and consequently produced *o*-chlorophenyl-β-gentiobioside were allowed to develop, but no glycoside could be detected in the resulting shoots or in the daughter corms (9). On the other hand, shoots

[1] The literature survey for this review was completed in July 1964.

[2] The following abbreviations will be used: ADPG [adenosine 5-(α-D-glucopyranosyl pyrophosphate)]; DOPamine (β-(3,4-dihydroxyphenyl)ethylamine]; PP (pyrophosphate); TDPG [thymidine 5-(α-D-glucopyranosyl pyrophosphate)]; TDPRha [thymidine 5-(L-rhamnopyranosyl pyrophosphate)]; THFA (tetrahydrofolic acid); UDP (uridine 5-pyrophosphate); UDPG [uridine 5-(α-D-glucopyranosyl pyrophosphate)]; UDPGA [uridine 5-(D-glucopyranosyluronic acid pyrophosphate)]; UDPRha (uridine 5-(L-rhamnopyranosyl pyrophosphate)]; UTP (uridine 5-triphosphate).

arising from *Vicia faba* seeds which had previously been treated with quinol contained the corresponding mono-β-D-glucopyranoside, arbutin (10). Macleod & Pridham (10) also showed that simple phenols and their glucosides and phenolic acids moved rapidly down the main stems of *V. faba* when they were fed in through the severed ends of the main veins of apical leaves or applied to laminae in dilute solutions of Teepol (an anionic detergent). There was some evidence to suggest that simple phenols were translocated less rapidly than the corresponding phenolic glucosides. An examination of the gut contents of colonies of *Macrosiphum pisi* feeding on the main stem showed that the phenols rapidly entered the phloem tissues. The aphids also contained DOPA after feeding on untreated bean plants, thus suggesting that this phenolic amino acid normally undergoes translocation. Similarly, studies with *Tuberolachnus salignes* (11) feeding on willow stems have indicated that low molecular weight phenolics (possibly cinnamic acid derivatives) may be present in the phloem, but so far none of these materials have been obtained in amounts sufficient for identification (10, 12). Observations in relation to the formation of high molecular weight phenolic compounds in wood and bark have been made by Hathway (13) and Hillis and his coworkers (14). Hathway, on the basis of girdling experiments with oak trees, concluded that the aglycones (+)-gallocatechin and leucodelphinidin are translocated to the cambium where they are oxidized to phlobotannins. Hillis, however, believes that little phenolic material is translocated in *Eucalyptus* but that the phenolic constituents of the wood are synthesized *in situ* from carbohydrates. His conclusions arise mainly from an examination of the constituents of various parts of the tree, and the results obtained by injecting D-glucose-^{14}C into kino veins. The observations of Wardrop & Cronshaw (15) support this latter theory.

It would appear that this controversy can only be solved by a careful examination of sieve-tube contents, and the most reliable method for doing this at present is the aphid-head technique, although, from a purely practical standpoint it is not an easy procedure.

In view of the ubiquitous nature of phenolic glycosides in the plant kingdom, it is perhaps pertinent to discuss the mechanism and importance of glycoside formation.

There is little doubt that phenols are phytotoxic agents. Some can interfere with oxidative phosphorylation (16) and the related phenomenon of mitosis (17); and Frey-Wyssling (18) has suggested a less specific toxicity arising from their surface-active nature and consequent interference with membrane function.

Glycosylation, it has often been suggested, is a detoxification mechanism analogous to glucuronic acid conjugation in animals. Toxic phenols can arise from normal plant metabolism and possibly by absorption from the soil [Whitehead (19) has shown that various soils contain phenolic acid derivatives]. The blocking of a phenolic hydroxyl group (or a carboxyl group of a phenolic acid) by the addition of a glycosyl residue can probably reduce

toxicity, although there is little direct evidence to support this hypothesis. *Vicia faba* seeds will, however, germinate and the plants develop to maturity in the presence of relatively large quantities of the glucoside arbutin, whereas the corresponding aglycone quinol, in equivalent concentrations, inhibits germination (20). Similarly, studies by Asen & Emsweller (21) with *Lilium* have shown that with an interspecific hybrid the ability to glucosylate ferulic (3-methoxy-4-hydroxycinnamic) acid is limited, and this probably accounts for the observed poor seed germination and the formation of tumors. On theoretical grounds, glycoslyation should destroy the surface-active properties of a phenol, and hence reduce the membrane interference suggested by Frey-Wyssling (18).

When foreign phenols are introduced into plants they are rapidly converted to the corresponding mono-β-D-glucopyranosides (22–24). This reaction is probably common to most angiosperms and gymnosperms and perhaps ferns; many bryophytes and algae however cannot glucosylate phenols (25). Both phenolic *O*-glucosides (26, 27) and glucose esters (27) are produced when hydroxycinnamic acids are fed to plant leaves. Phenolic *O*-glucosides are present in most nonwoody tissues, and a number of other monosaccharides including L-rhamnose, D-galactose, D-glucuronic acid, and L-arabinose are also frequently found in combination with phenols. In addition, combined disaccharides [e.g., rutinose (6-*O*-α-L-rhamnopyranosyl-D-glucopyranose) and sophorose (2-*O*-β-D-glucopyranosyl-D-glucopyranose)] and trisaccharides are of common occurrence, and it is probable that even more complex glycosyl derivatives remain at present undetected in plants. The nature of the known glycosyl residues and the biochemical pathways leading to their formation have been reviewed in detail by Pridham (28).

Cardini & Leloir (29) first showed that arbutin could be synthesized by incubating quinol with UDPG in the presence of wheat germ extracts. These results were later confirmed and extended by Pridham & Saltmarsh (24, 30) using enzyme preparations from *Vicia faba* seeds and wheat germ. The following generalized reactions were demonstrated with these preparations:

$$\text{UTP} + \alpha\text{-D-glucose-1-phosphate} \rightarrow \text{UDPG} + \text{PP}$$

$$\text{UDPG} + \text{Ar-OH} \rightarrow \text{Ar-O-G} + \text{UDP}$$

The UDPG:phenol glucosyltransferase activity present in wheat germ has been studied in some detail by Yamaha & Cardini (31). An enzyme specific for the monoglucosylation of phenols with 1,4-dihydroxyl groups appears to be present. Mung bean preparations, together with UDPG or TDPG, have been used by Barber (32) to glucosylate the flavonol, quercetin (probably in the 3-position). ADPG is stated to be a better glucosyl donor than UDPG in the presence of quinol and a wheat germ extract (33).

Enzymic transfer of glucose from UDPG to carboxylic acid groups has also been demonstrated *in vitro* (34), and it is probable that the naturally occurring glucose esters of hydroxybenzoic and cinnamic acids are formed by a similar mechanism.

β-Glucosidase (β-D-glucoside glucohydrolase) can catalyse the transfer of glucosyl residues to phenolic *O*-glucosides with the formation of *O*-diglucosides. Thus, when arbutin is incubated with this enzyme, the glucoside serves as both a glucosyl donor and acceptor, and *p*-hydroxyphenyl-β-gentiobioside results (35, 36). Nucleoside diphosphate sugars are again the more likely donors for phenolic *O*-diglycoside formation *in vivo*. Cardini & Yamaha (37) have shown that wheat germ possesses an enzyme which brings about the glucosylation of arbutin in the presence of UDPG. The product is again the β-gentiobioside, and the enzyme seems to be distinct from the one that catalyses the glucosylation of quinol (31).

Nucleoside diphosphate derivatives of monosaccharides other than glucose may also be involved in the biosynthesis of glycosides. Marsh (38), for example, has prepared a β-D-glucuronoside of quercetin by incubating a *Phaseolus vulgaris* preparation with UDPGA and the flavonol. Quercetin-3-glucosiduronic acid occurs naturally in *P. vulgaris* (39). TDPRha has been used as an L-rhamnose donor for the synthesis of a β-rutinoside of quercetin using quercetin-3-glucoside as an acceptor and, again, a *P. vulgaris* enzyme (32). The product is probably identical with the common plant glycoside, rutin. Barber (40) believes that *in vivo*, the L-rhamnose donor is more likely to be UDPRha, however.

In conclusion, it seems very probable that nucleoside diphosphate sugar derivatives are involved in the biosynthesis of phenolic glycosides. Points in favor of this hypothesis are: (*a*) most of the necessary nucleotide derivatives appear to be present in plant tissues (41); (*b*) this type of transfer reaction is energetically favorable and is coupled to the general metabolism of the plant; and (*c*) nucleoside diphosphate sugar derivatives function as phenol glycosylating agents *in vitro*.

Up to the present time, attempts to synthesize phenolic β-D-glycosides[3] using higher plant enzymes and "low energy" monosaccharide donors have failed (24, 36, 37, 42), although phenolic α-D-glucosides have been prepared using an *Aspergillus niger* enzyme and maltose as the donor (43). Pridham & Wallenfels (44) have used *E. coli* β-galactoside (β-D-galactoside galactohydrolase) to synthesize *m*-hydroxyphenyl-β-D-galactoside and -β-D-fucoside from resorcinol using lactose and *o*-nitrophenyl-β-D-fucoside, respectively, as donor molecules. Recently, Nakamura & Miwa (45) have utilized the energy of the glycosidic bond in sucrose to produce *p*-hydroxyphenyl-β-D-fructofuranoside using invertase (β-D-fructofuranoside fructohydrolase) preparations from both microorganisms and wheat.

With regard to the acceptors for glycosylation reactions *in vivo*, it is generally assumed that the first glycosyl residue is transferred to the phenol at a late stage of the biosynthesis. In the case of more complex phenols, such as the flavonoids, glycosylation of a hydroxyl group could conceivably occur

[3] D- and L-glycosyl residues in naturally occurring phenolic glycosides normally have β and α configurations, respectively, at the anomeric carbons.

just prior to the completion of the flavonoid skeleton. Thus, the pathway demonstrated by Barber (32) is not necessarily the one which occurs *in vivo*. With compounds like rutin which have complex glycosyl residues it is very probable that single glycosyl residues are added stepwise. Support for this idea comes from studies of the co-occurrence of related glycosides within a single species (e.g., 46–49).

In addition to detoxification, glycoside formation may be important in a number of other ways. For example, it leads to a general decrease in chemical reactivity of the phenol, particularly towards enzymic and aerobic oxidation (50). Thus, anthocyanidins are much less stable than anthocyanidin-3-glycosides. Glycosylation also leads to increased sap solubility, and this is presumably very important in the case of high molecular weight phenols such as the flavonoids (51). The importance of phenolic glycosides as stores of carbohydrate is in dispute. The concentrations of these compounds in the tissues do show both long and short-term periodic variations, but there is really little evidence at present to suggest that they are hydrolysed or form important respiratory substrates in normal healthy plants. Much of the early work on this subject has been reviewed by Haas & Hill (52) and Pridham (20). Some glycosides may act as latent antimicrobic substances which are hydrolyzed when cell necrosis occurs (by hydrolases from the disrupted plant cells or from an attacking parasite) with the consequent liberation of toxic phenols.

There is now a growing body of evidence which suggests that at least some phenolic glycosides are important plant metabolites. For example, glucosides of cinnamic acids appear to be involved in the biosynthesis of coumarins (4) and glucosides of cinnamyl alcohols possibly in lignin formation (6, 53). Flavonoid glycosides may control indole-3-acetic acid oxidase activity; *in vitro* quercetin derivatives act as inhibitors and kaempferol derivatives as activators (54). The importance of the complex glycosyl residues which are bonded to some phenols is puzzling. It is possible that they are related in some way to the biological reactivity of the aglycone and perhaps only allow interaction with specific enzymes. Another interesting possibility is that glycosides of potential quinones might be involved in a process of oxidative transglucosylation. Thus, Cardini (55) demonstrated the formation of gentiobiose and higher oligosaccharides when arbutin was treated with bromine. No oligosaccharides were observed when bromine was replaced by a potato phenolase preparation, however. Oxidative transglucosylation would be analogous to the hypothetical mechanism proposed for oxidative phosphorylation involving quinol phosphates [e.g., Harrison (56); Clark, Hutchinson & Todd (57)].

Shikimic Acid Pathway

The major pathway for the biosynthesis of monocyclic aromatic compounds undoubtedly involves shikimic acid. This pathway, which is summarized as far as the aromatic amino acids in Figure 1, was largely con-

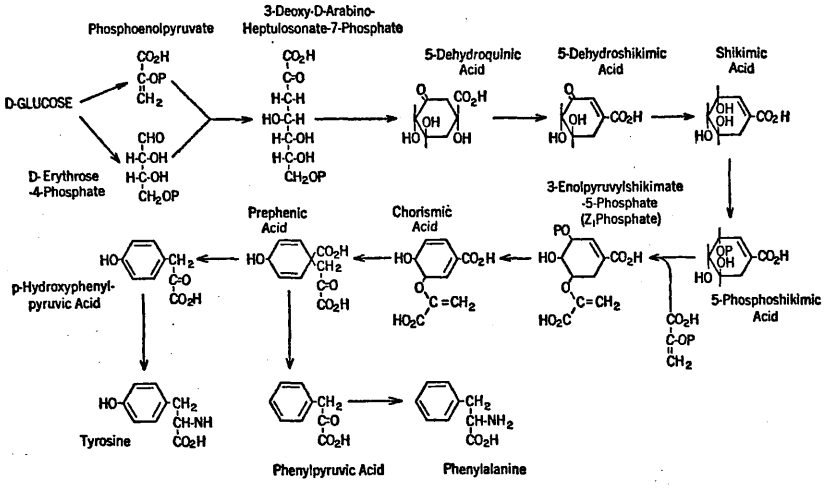


FIG. 1. The shikimic acid pathway.

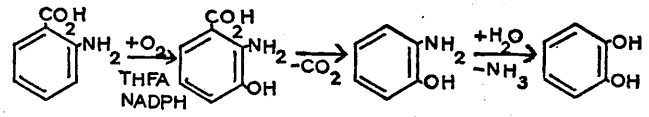
structed from studies with mutant microorganisms, although the basic sequence of reactions is now generally believed to be responsible for the formation of L-phenylalanine and L-tyrosine, and hence other aromatic derivatives in higher plants. Many of the individual steps in plants have yet to be demonstrated conclusively, however. Reviews by Neish (3), Geissman (58), and Higuchi & Kawamura (59), for example, have covered the work leading up to the elucidation of the main shikimic acid pathway. Recent discoveries of pathway details include those of Levin & Sprinson (60) who have shown that *E. coli* preparations can catalyse the formation of 3-enolpyruvylshikimate-5-phosphate (from phosphoenol pyruvate and 5-phosphoshikimic acid). Gibson and his collaborators (61, 62), using *Aerobacter aerogenes*, have detected another intermediate, chorismic acid, which is formed from 3-enolpyruvylshikimate-5-phosphate and can be converted enzymically to prephenic acid. Returning to higher plants, Gamborg & Simpson (63) have demonstrated that the enzyme complex necessary for the conversion of prephenic acid to phenylalanine and tyrosine is present in *Phaseolus vulgaris* shoots. The complex presumably contains prephenic aromatase and dehydrogenase and the necessary transaminase(s). Thus when prephenate was incubated with L-glutamic acid, NADP, and the *Phaseolus* preparation, phenylalanine and tyrosine were formed. If NADP was replaced by NAD, only phenylalanine was produced. In the absence of the glutamate, the keto acid intermediates accumulated.

SIMPLE PHENOLS

Hydroxybenzenes and their methylated derivatives, as judged by literature reports (64), are not particularly common in higher plants. Quinol, com-

bined as the glucoside arbutin, is one of the better known compounds, but it appears to be confined mainly to the Ericaceae and *Pyrus* (65). Hattori & Satô (66) have shown that chloroplasts from *Pyrus*, *Pyrola*, and *Saxifraga* species can oxidize arbutin by a tyrosinase-type reaction to a number of products, one of which has been tentatively identified as 3,4-dihydroxyphenyl β-D-glucoside. [This compound is also produced when 1,2,4-trihydroxybenzene is fed to germinating *Vicia faba* seeds (24).] Quinol itself is not oxidized by the chloroplasts, and the Japanese workers suggest that the blackening of *Pyrus* leaves is a result of the direct oxidation of arbutin.

Catechol is less widely distributed in the plant kingdom than quinol. It occurs in grapefruit tissues (67) and in the Salicaceae (65), and recently Madhusudanan Nair & Vaidyanathan (68) have reported that anthranilic acid [which is formed via the shikimic acid pathway in bacteria (60)] can be converted to this dihydric phenol by a chloroplastic enzyme system from the leaves of *Tecoma stans*. The system has also been obtained in a soluble form (69) and fractionated into three components by adsorption on alumina and calcium phosphate gels. The overall reaction appears to be:



An anthranilic acid oxidase system has also been observed in tryptophan-adapted strains of *Pseudomonas fluorescens* (70).

Cinnamic Acids

During the last fifteen years, more attention has been focussed on the hydroxycinnamic acids than perhaps any other group of naturally occurring phenols. Interests have centered mainly around the part played by these compounds and their derivatives in disease resistance and in the biosynthesis of coumarins, flavonoids, and lignin. In addition, biologists have studied the distribution of the cinnamic acids with a view to extending the science of chemical taxonomy. Members of this group of compounds are common constituents of plant tissues where they occur as esters of quinic acid (64, 71), shikimic acid (72), and glucose, and as phenolic glycosides (26). The acids *p*-coumaric (4-hydroxycinnamic), caffeic (3,4-dihydroxycinnamic), ferulic (3-methoxy-4-hydroxycinnamic), and sinapic (3,5-dimethoxy-4-hydroxycinnamic) are frequently reported as constituents of many different species. Other naturally occurring compounds which are less often encountered in the literature include cinnamic acid itself (64), *o*-coumaric (2-hydroxycinnamic) (64), *p*-methoxycinnamic (73), orthoferulic (2-hydroxy-3-methoxycinnamic) (74), isoferulic (3-hydroxy-4-methoxycinnamic) (64, 75), and 3,4,5-trimethoxycinnamic acids (73). Some of these are not easy to detect, however, and so it is difficult to state at present whether or not they are rare tissue components.

An outline of the formation and metabolism of the common cinnamic acid derivatives is given in Figure 2. The pathways have been deduced mainly by feeding experiments with ^{14}C-labelled compounds; the greater part of these studies up to 1961 has been explained and discussed by various workers, including Neish (3) and Brown (5). No detailed repetition of the contents of these reviews is intended here. It should, however, be noted at this point that the major pathway for the formation of the cinnamic acids is via prephenic and the aromatic amino acids, and that cinnamic acid itself can arise directly from phenylalanine by the action of L-phenylalanine ammonia lyase (phenylalanase) (76). Similarly, *p*-coumaric acid can be produced directly from tyrosine by L-tyrosine ammonia lyase (tyrase) (77). This latter enzyme appears to be prominent only in the grasses, although other plants probably possess some tyrase activity (78). A third deaminase activity has been reported to occur in *Taraxacum* and *Hordeum* (79). Extracts of acetone powders from these and other species can remove ammonia from DOPA with the formation of caffeic acid. This reaction may be catalysed by phenylalanase or tyrase, or a separate enzyme may be involved.

It was originally postulated that β-phenyllactic and β-(*p*-hydroxyphenyl)-lactic acids are intermediates in the conversion of phenylalanine and tyrosine to cinnamic and *p*-coumaric acids, respectively (80). This idea was based on the ready utilization of the lactic acid derivatives for lignin formation. Gamborg, Wetter & Neish (81) have, however, shown that these acids can be converted to the corresponding pyruvic acid derivatives by glycollate:O_2 oxidoreductase (glycollate oxidase) from a number of different species. Phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid could then form phenylalanine and tyrosine, respectively, in the presence of the appropriate transaminases. These latter enzymes are present in higher plant tissues, and their reactions are reversible (63, 82, 83). This overall mechanism could then account for the incorporation of ^{14}C from the lactic acid derivatives into lignin, but Gamborg, Wetter & Neish (81) consider this to be a side reaction and state that, "there is now no reason to believe that α-hydroxy acids are natural intermediates in aromatic acid metabolism." More recent studies by Higuchi & Brown (84) with wheat plants, using isotope trapping and competition techniques, indicate that phenylalanine and tyrosine can to some degree be converted to β-phenyllactic and β-(*p*-hydroxyphenyl)lactic acids, respectively; thus the formation of cinnamic and *p*-coumaric acids by dehydration of these α-hydroxy acids still remains a possibility. Higuchi & Brown suggest, however, that if such a pathway does exist it is only a minor one.

Another important contribution to our understanding of the biosynthesis of hydroxycinnamic acids was made by McCalla & Neish (85). Using *Salvia splendens*, they studied the kinetics of the formation of these compounds from ^{14}C-labelled phenylalanine and concluded that the pathway from cinnamic to sinapic acid (excluding 5-hydroxyferulic acid) consisted of a series of hydroxylation and methylation reactions as shown in Figure 2. Probably *o*-coumaric acid is also formed by orthohydroxylation of cinnamic acid (86–88). In addi-

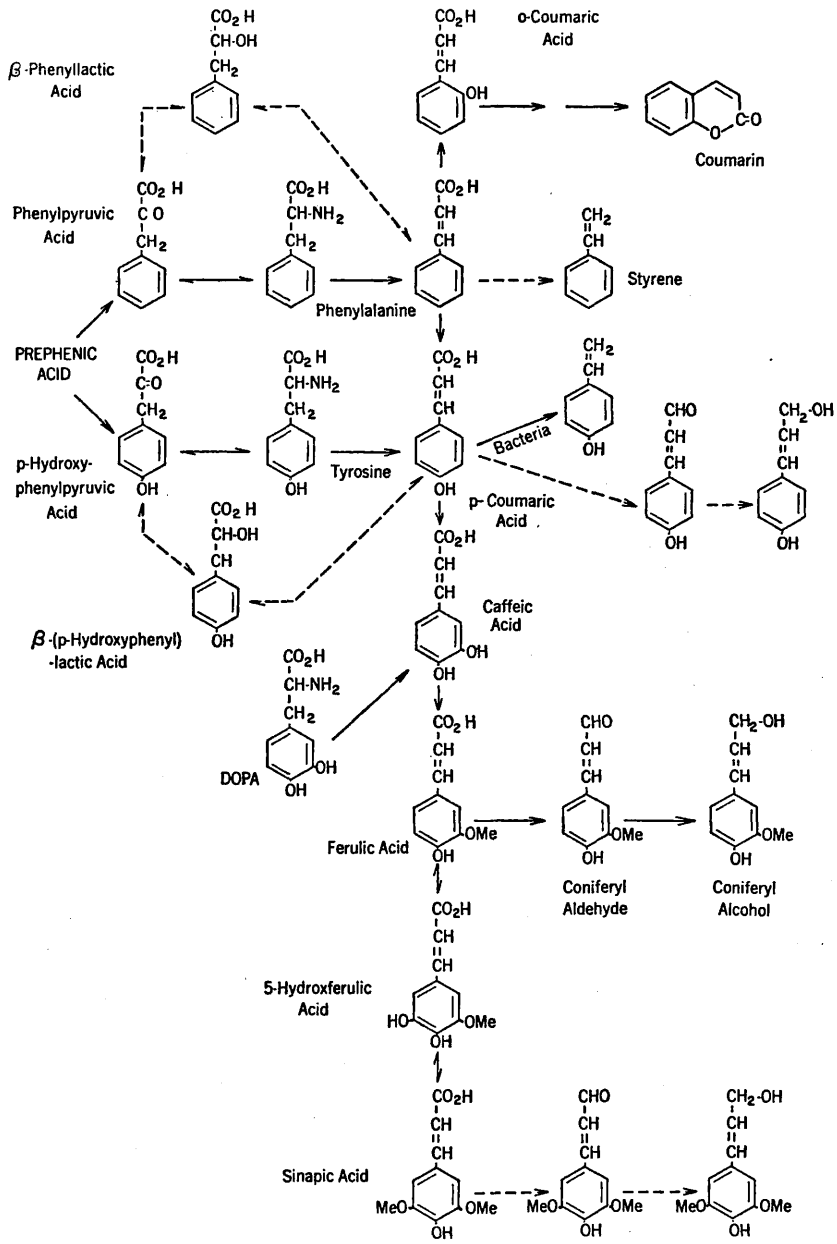
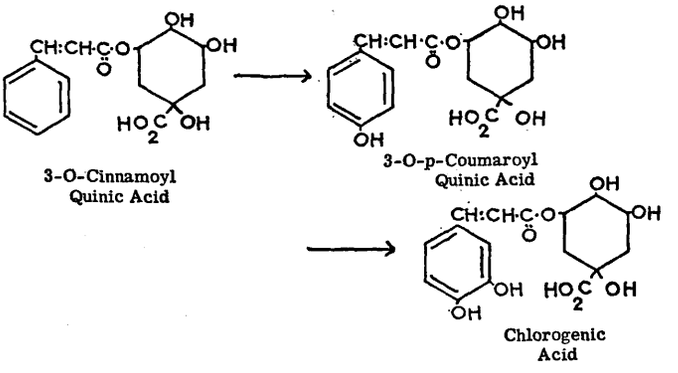


FIG. 2. The biosynthesis and metabolism of cinnamic acids. Arrows with broken lines indicate hypothetical or probable minor pathways.

tion, Levy & Zucker (89), working with potato, suggest that a hydroxylation mechanism is in operation for the formation of chlorogenic acid, the intermediates in this case being bound as quinate esters:



Bound rather than free cinnamic acids may always be the forms which are metabolically important in plants. In addition to esters of cyclohexane carboxylic acids, glucose and coenzyme A esters [cf. Grisebach & Vollmer (87)] could also be involved in reactions such as those described above. The studies of McCalla & Neish (85) with *Salvia* showed that the series of reactions from cinnamic to sinapic acid are not readily reversible although there is some incorporation of label into simpler members of the series when more complex members are fed. In this connection a dehydroxylation reaction has recently been recorded by El-Basyouni et al. (88) who observed that caffeic acid is converted to *p*-hydroxybenzoic by *Triticum* presumably by removal of a hydroxyl group followed by *β*-oxidation. Several examples of *O*-demethylation [e.g., Reznik & Urban (90)], and demethoxylation [e.g., Kratzl (91); Higuchi & Brown (92); El-Basyouni et al. (88)] reactions by plants are to be found in the literature. There is no reason to suppose that demethoxylation can be achieved biochemically in a single step; instead it is probably demethylation followed by dehydroxylation. Thus, the conversion of sinapic acid to ferulic acid by wheat plants presumably occurs as follows (92):

sinapic acid → 5-hydroxyferulic acid → ferulic acid

This system appears to be reversible, but the extent is dependent on the age of the plant. No information is available on the occurrence of 3,4,5-trihydroxycinnamic acid which would result from the demethylation of 5-hydroxyferulic acid or the hydroxylation of caffeic acid, although Zenk (93) believes that this compound is a precursor of gallic acid.

Higuchi & Brown (84, 92) have shown that ferulic acid-2-^{14}C can be reduced to coniferyl (3-methoxy-4-hydroxycinnamyl) alcohol via coniferyl aldehyde by heading wheat plants and cambial tissue from *Pinus strobus*.

Preliminary experiments with acetone powders have provided additional evidence for the reduction of ferulic acid to coniferyl aldehyde (94). The two other cinnamyl alcohols (*p*-hydroxy- and 3,5-dimethoxy-4-hydroxy-cinnamyl alcohols), which Freudenberg also believes are important lignin precursors (6, 53), could be derived from the corresponding cinnamic acids. Regarding the participation of coniferin (4-*O*-β-D-glucosylconiferyl alcohol) in lignification, Higuchi & Brown have suggested that this glucoside is a storage product which is only formed by a few plant species and that it is not directly involved.

Studies with *Aerobacter aerogenes* (95) have revealed a decarboxylase which can convert hydroxycinnamic acids to the corresponding hydroxystyrene derivatives. It is therefore possible that *p*-hydroxystyrene, which is found, for example, in *Viburnum furcatum* (96), may be formed from *p*-coumaric acid. Similarly, styrene itself might be produced from cinnamic acid. Finkle and his associates (95) have noted the co-occurrence of these latter two compounds in *Styrax* and other species.

In view of the recent and very full account of the biosynthesis of natural coumarins by Brown (4), only those advances which have been made in this field during the last few months will be mentioned here. Coumarin and derivatives of coumarin with hydroxyl and methoxyl groups on the benzene ring are generally derived in higher plants by cyclization of cinnamic acids with related structures. Some of the details of these reactions are in dispute; for example, the role of coumarinyl glucoside (2-glucosyloxy-*cis*-cinnamic acid) in coumarin formation. However, a general reaction pattern does seem to be emerging for the biosynthesis of this group of compounds. A detailed study has now been made of the formation of umbelliferone (7-hydroxycoumarin) in *Hydrangea macrophylla* (97). The ^{14}C-labelled *p*-coumaric and *o*-coumaric acids are incorporated efficiently into umbelliferone, both probably via umbellic (2,4-dihydroxycinnamic) acid. *Hydrangea* cannot, however, ortho-hydroxylate cinnamic acid but will convert the latter to *p*-coumaric acid and umbelliferone. Thus, *o*-coumaric acid may not function as a precursor *in vivo* but the following pathway is likely to exist:

cinnamic acid → *p*-coumaric acid → umbellic acid → umbelliferone

There appears to be very little free umbelliferone in *Hydrangea*, and hydrolysis studies suggest that it may be present as *cis*-2-glucosyloxy-4-hydroxycinnamic acid by analogy with the bound forms of coumarin and herniarin which occur naturally (4). Initial experiments on the formation of hydrangetin (7-methoxy-8-hydroxycoumarin, which is also present in *Hydrangea*) have shown that both cinnamic and *p*-coumaric acids can serve as precursors (97). The detection of orthoferulic acid in wheat shoots may indicate that this compound is a precursor of 8-methoxycoumarin which is also a constituent of this plant (74).

Two pathways appear to exist for the biosynthesis of coumarin. One involves the utilization of transcinnamic acid via the glucosides of *o*-coumaric

(*trans-o*-hydroxycinnamic) and coumarinic (*cis-o*-hydroxycinnamic) acids (86, 98, 99) and the other, *cis*-cinnamic acid which is probably hydroxylated to coumarinic acid which in turn lactonizes (100). The *o*-coumaric acid β-D-glucoside is converted to coumarin by extracts of *Melilotus alba*, thus showing that plants do contain a *cis-trans* isomerase system (101).

The cinnamic acids are obviously important metabolites in plant tissues (their role in the formation of hydroxybenzoic acids is discussed in the next section), but in addition they may also be involved in regulatory mechanisms in the cell (102). Thus, Rabin & Klein (103) have shown that caffeic and chlorogenic acids are inhibitors of indole-3-acetic acid oxidase. Esterified *p*-coumaric acid occurs in pineapple stems where it appears to act as an oxidase stimulator. Esterified ferulic acid which is also present is a stimulator at low concentrations but at higher levels is a powerful inhibitor (104). It is interesting to note that other phenolic compounds with *o*-dihydroxyl or *o*-hydroxymethoxyl groups are commonly inhibitors of indole-3-acetic acid oxidase, whereas compounds possessing a *p*-hydroxyphenyl group are activators (54, 105, 106). Similar rules apply to phenolic compounds which govern the rate of oxidation of NADH by horseradish peroxidase and hydrogen peroxide (107). In this instance *p*-coumaric acid behaves as an activator and ferulic and sinapic acids as competitive inhibitors. The results obtained in this study by Gamborg, Wetter & Neish (107) showed that there is a close similarity between the peroxidase and indole-3-acetic oxidase systems. The possibility that the two systems are identical was raised.

An enzyme not involved in phenol metabolism, potato phosphorylase (α-1,4-glucan:orthophosphate glucosyl transferase), is also strongly inhibited by chlorogenic acid (108). It should be stressed that all the information on enzyme regulation by phenolic compounds is derived from *in vitro* observations, and at present there is no evidence either for or against these activation and inhibition phenomena occurring *in vivo*.

Hydroxybenzoic Acids

Hydroxybenzoic acids and closely related reduced and methylated derivatives are found in most higher plant tissues (64, 65, 109–114). Paper chromatographic studies suggest that *p*-hydroxybenzoic, protocatechuic (3,4-dihydroxybenzoic), gentisic (2,5-dihydroxybenzoic), and vanillic (3-methoxy-4-hydroxybenzoic) acids (65, 110, 114) occur most commonly; Ibrahim, Towers & Gibbs (114) have discussed the taxonomic significance of the group.

The majority of these compounds probably arise via the shikimic acid pathway. Early studies with microorganisms gave the first clues regarding their origin. For example, it was shown that protocatechuic acid is formed from 5-dehydroshikimic acid by dehydration using mutant strains of *Neurospora crassa* (115). Protocatechuic acid and gallic acid (3,4,5-trihydroxybenzoic acid) are formed when *Phycomyces blakesleeanus* is grown on a glucose medium (116). Haslam, Haworth & Knowles (117) have measured the

amounts of gallic acid formed on feeding various potential precursors to this mould and the results suggest that the following pathway is operative:

5-dehydroquinic acid → 5-dehydroshikimic acids → gallic acid

These workers did not believe that gallic acid was formed from protocatechuic acid by hydroxylation because (*a*) ^{14}C-labelled protocatechuic acid did not give rise to labelled gallic acid when administered to *Phycomyces* cultures, and (*b*) they were unable to hydroxylate protocatechuic acid with a phenolase preparation.

It is believed that *p*-hydroxybenzoic acid is derived from shikimic acid in *Penicillum patulum* (118), and recently it has been shown that chorismic acid can be converted enzymically to *p*-hydroxybenzoic acid (119).

Up to the present time there has been little to suggest that dirct pathways from nonaromatic precursors to hydroxybenzoic acids, as found in microorganisms, are important in higher plants. Conn & Swain (120) have, however, shown that such reactions may occur in *Geranium pyrenaicum* which can convert glucose much more efficiently than phenylalanine to gallic acid. The possibility that β-oxidation of cinnamic acid derivatives might form an alternative route to hydroxybenzoic acids was suggested by Geissman & Hinreiner (121), and Henderson & Farmer (122) showed that ferulic acid could be converted to vanillic acid by soil fungi. Feeding experiments using ^{14}C-labelled glucose and phenylalanine led Zenk (93) to the conclusion that β-oxidation of 3,4,5-trihydroxycinnamic acid is a likely reaction for the formation of gallic acid in *Rhus typhina*. Conn & Swain (120) had noted this possibility, as labelled phenylalanine, when fed to *Geranium*, had also been incorporated into the flavonoid myricetin which has a vicinal trihydroxy grouping in the B ring. In the case of *Gaultheria procumbens*, treatment with cinnamic acid-3-^{14}C yielded methysalicylate with a labelled carboxyl carbon atom (87). A detailed study of this alternative pathway by El-Basyouni et al. (88) using a number of different di- and monocotyledenous plants strongly supports Geissman & Hinreiner's hypothesis (121). Removal of a 2-carbon fragment was observed when *p*-coumaric, ferulic, caffeic, and sinapic acids (all ^{14}C-labelled in the β-position) were fed to leaf discs or shoots with the formation of the corresponding hydroxy- or hydroxymethoxy-benzoic acids (see Fig. 3). The formation of several labelled orthohydroxylated acids by L-phenylalanine-U-^{14}C and cinnamic acid-3-^{14}C-treated plants also suggested that *o*-coumaric acid was a precursor of salicylic acid [cf. Grisebach & Vollmer (87)]. Gross & Schutte (123) observed that the benzoic acid moiety of cocaine became labelled when phenylalanine-3-^{14}C was administered to *Erythroxylon novogranatense*. This acid which occurs in the free state in plant resins and also as a glucose derivative, vaccinin, in cranberries (124) may be produced by the degradation of cinnamic acid. Feeding experiments carried out by Zenk & Miller (125) with *Catalpa ovata* confirm this and the formation of *p*-hydroxybenzoic acid by β-oxidation of *p*-coumaric acid.

A second and equally important mechanism for the formation of hydroxy-

benzoic acids appears to be the hydroxylation and *O*-methylation of existing C_6-C_1 compounds. Thus, Ibrahim & Towers (111) in 1959 reported that plants treated with benzoic acid-^{14}C produced labelled salicylic and *p*-hydroxybenzoic acids. Klämbt (126) also showed that *Helianthus* hypocotyls can convert benzoic acid to salicylic acid and 2-β-D-glucosyloxybenzoic acid. Leaves of various species including *Gaultheria procumbens* and *Lotus arabicus* were able, according to Towers & Ibrahim (111), to hydroxylate salicyclic acid with the formation of gentisic and *o*-pyrocatechuic (2,3-dihydroxybenzoic) acids. Some small conversion of *p*-hydroxybenzoic acid to proto-

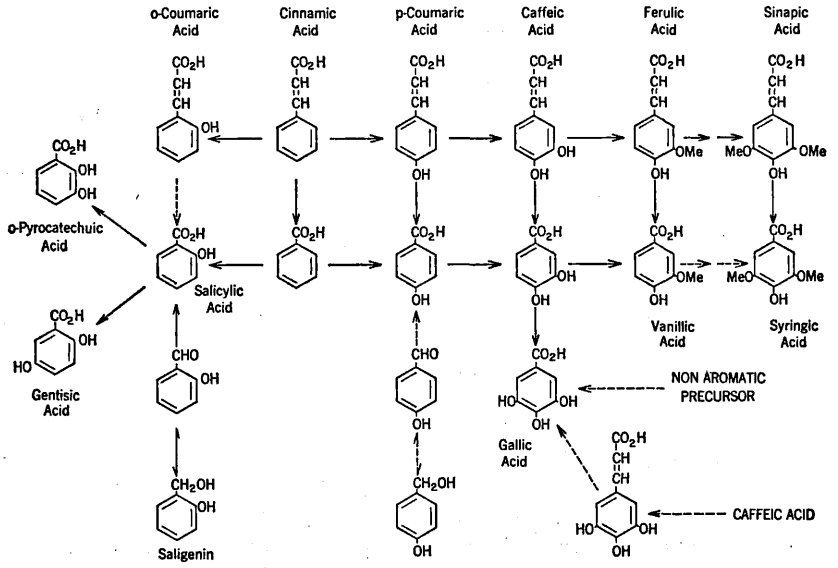


FIG. 3. Biogenetic relationships between naturally occurring hydroxybenzoic acids and related derivatives. Arrows with broken lines indicate hypothetical or probable minor pathways.

catechuic acid has also been observed, and the latter compound itself may undergo further reaction, the nature of which depends on the plant species being used. For example, protocatechuic acid is converted to gallic acid by *Pelargonium*, but with *Hordeum* and *Oryza* the dihydric phenol is methylated to form vanillic acid (88). The danger of constructing a metabolic pathway based on observations with one species thus becomes apparent. Another possible pitfall is the use of tissues of different ages. In this connection El-Basyouni and his associates (88) noted that older leaf tissues (6 weeks) lost much of their ability to ortho- and para-hydroxylate benzoic acid in comparison with tissues 2 to 3 weeks old. Vanillic acid would appear to be metabolically stable since introduction of this compound (^{14}C-labelled) into leaf segments of four different species yielded no other radioactive phenols (88).

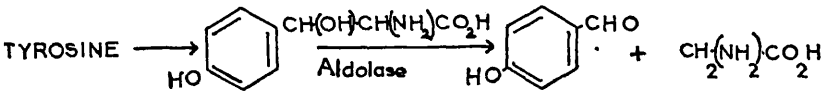
Hydroxylation and *O*-methylation of benzoic and hydroxybenzoic acid derivatives *in vitro* are, therefore, well established, but what enzymes are concerned with the individual reactions? Methionine, as an *S*-adenosyl derivative, is undoubtedly an important methyl donor for methylation reactions [cf. Byrrum et al. (127); Bohm & Towers (113)]. The discovery by Finkle and co-workers (128) of *O*-methyl transferase activity in cambial scrapings from apple and *Pittosporum crassifolia* and in *Cortaderia selloana* shoots (129) is confirmation of this widely accepted hypothesis. Probably less is known about hydroxylation reactions *in vivo*. The phenolase system will introduce *in vitro* a hydroxyl group *ortho* to an existing phenolic hydroxyl and it is, for example, generally believed to be responsible for the conversion of tyrosine to DOPA. A crude phenolase preparation from potato will also form protocatechuic acid from *p*-hydroxybenzoic acid (117). Little other relevant information is available, however, regarding the role of phenolase in biosynthesis, and many questions remain to be answered. For example, what mechanism is involved in the hydroxylation of benzoic acid to salicylic acid? An interesting model hydroxylating system has been studied by Buhler & Mason (130) using purified horseradish peroxidase in the presence of dihydroxyfumarate and oxygen. This system effects many of the hydroxylations which are observed when phenolic acids are introduced into plant tissues. Thus, benzoic acid can be converted to salicylic and *p*-hydroxybenzoic acids and salicylic acid to *o*-pyrocatechuic and gentisic acids. No reaction occurs if oxygen is replaced by hydrogen peroxide; thus, the peroxidase is behaving as an oxidase in the system. Buhler & Mason make no comment on the possible biochemical significance of these reactions. If a similar system does function in the plant, one would expect the dihydroxyfumarate to be replaced by a suitable, naturally occurring cofactor. The obvious replacement, ascorbic acid, is, however, ineffective.

Reduction products of phenolic carboxylic acids also occur in plants and appear to be metabolically active. Pridham & Young (131), using *Vicia faba* seedlings, demonstrated the interconversion of saligenin (*o*-hydroxybenzyl alcohol) and salicylaldehyde and the oxidation of the latter to salicylic acid. Reduction of salicylic acid was not observed, although *Neurospora crassa* can achieve this reduction and produce saligenin (132). It is possible that salicin (*o*-hydroxymethylphenyl β-D-glucopyranoside) is formed *in vivo* by reduction of helicin (*o*-formylphenyl β-D-glucopyranoside) and not by the direct glucosylation of saligenin. Feeding the latter to plants mainly produces the isomer of salicin (*o*-hydroxybenzyl β-D-glucopyranoside), which is not a natural plant constituent (24).

p-Hydroxybenzyl alcohol has attracted attention in that it appears to be a naturally occurring cofactor for indole-3-acetic acid oxidase (119). It may well be on a pathway (analogous to that of saligenin) to *p*-hydroxybenzoic acid via *p*-hydroxybenzaldehyde. The latter compound has been detected in the vanilla bean and in *Papaver somniferum* (64).

Neish (3) has drawn attention to the possibility of aromatic aldehyde

formation in plants from phenylpropanoid derivatives in the presence of a phenylserine aldolase similar to that found in liver by Bruns & Fiedler (133). A possible pathway to *p*-hydroxybenzaldehyde might, therefore, be:



Some support for the first step in this reaction comes from a report by Gander (134) that both intact plants and cell-free extracts can convert tyrosine to 3-(*p*-hydroxyphenyl)serine.

Phenolic Amino Acids and Amines

The large number of different phenolic amines, and to a lesser extent phenolic amino acids, which occur in higher plants is perhaps not generally appreciated, although the pharmacological action and metabolism of these compounds in animals has been extensively investigated. In the plant all the monocylic phenolic amines are probably derived from prephenic acid via tyrosine, the latter being widely distributed in the plant kingdom in both free and combined forms. The pathway to the 3,4-dihydroxyphenylalkylamines may, at least in some species, involve DOPA. This amino acid is less common than others and is confined mainly to leguminous plants [e.g., *Vicia* (135, 136); *Stizolobium* (137); *Baptisia* (136)] and a few other species such as *Aristolochia clematilis* (138), *Morus alba* (139), and *Euphorbia lathyrus* (140). It is a nonprotein amino acid which generally occurs in the free state, sometimes in relatively high concentrations [e.g., *Vicia faba* (135)], and it is usually associated with melanin formation and consequent tissue blackening. In the case of the broad bean, Nagasawa and his associates (141) detected a glucoside of DOPA in the green seed tests. Andrews & Pridham (142) have investigated the structure of this compound and have shown that it is the 3-*O*-β-D-glucopyranoside. The same derivative is formed when DOPA is fed to germinating *Pisum sativa* seeds. As stated in the section on glycosidic derivatives, phenols generally occur in plants in combination with sugars. DOPA, however, appears to be an exception to this rule in some cases.

In *Vicia faba* leaves, there is no detectable DOPA glucoside, but there is a relatively high concentration of the aglycone. The absence of a specific enzyme for glucose transfer in the leaves may account for this, or if the enzyme is present, it may not come into contact with DOPA. The precursor of DOPA is, no doubt, tyrosine which probably undergoes direct hydroxylation catalysed by phenolase. Experiments by Evans & Raper (143) as early as 1937 proved that this conversion could be effected by potato and wheat bran preparations possessing tyrosinase activity. Liss (144), using *Euphorbia lathyrus*, has shown that labelled DOPA is formed from tyrosine-^{14}C. Little detailed work has been carried out on this reaction. Crude enzyme preparations have normally been used, and the possibility of other biosynthetic path-

ways occurring *in vivo* should not be excluded. For example, an indirect route from tyrosine to DOPA as follows could conceivably exist:

tyrosine → *p*-hydroxyphenylpyruvic → 3,4-dihydroxyphenylpyruvic acid → DOPA

Furthermore, the complex relationships which must exist between tyrosine, DOPA, and phenolase require investigation, particularly in view of the ubiquitous nature of the phenolase system and the common occurrence of tyrosine in plants as opposed to the apparently limited distribution of DOPA. In the case of *Vicia faba*, where the DOPA content is high, one contributing factor could be the low DOPA ammonia lyase activity which is present in the leaves of this plant (79). On the other hand, from the point of view of DOPA formation, much of the phenolase in broad bean may be in a latent form in the tissues (145).

Other phenolic amino acids which have been detected in higher plants are *N*-methyltyrosine (146), which is a constituent of several species, and very recently, D-(3-carboxy-4-hydroxyphenyl) glycine and L-(3-carboxy-4-hydroxyphenyl)alanine in *Reseda luteola* seeds (147). The hydroxyphenylalkylamines and examples of plants in which they occur are listed in Table I.

Ideas regarding the pathways for the formation of these various amine derivatives often come from an inspection of the compounds which occur together in a single plant species. Thus tyrosine, tyramine, DOPA, DOPamine, and epinine are found in *Sarothamnus scoparius* (148, 157), and this immediately suggests two possible routes for the formation of epinine from tyrosine:

tyrosine → tyramine → DOPamine → epinine 1.

tyrosine → DOPA → DOPamine → epinine 2.

Correale & Cortese (166), using suspensions of ground *Sarothamnus* seedlings for *in vitro* studies, suggested that the first was the more likely pathway. They demonstrated that the seedling preparation could decarboxylate tyrosine with the formation of tyramine (5 per cent conversion), and this in turn could be hydroxylated to DOPamine (2 per cent conversion). A final stage, *N*-methylation, which was not investigated, would give rise to epinine. Jaminet (167) also believes that tyramine is the direct precursor of DOPamine in *Sarothamnus*, and he is supported by Buckley & Towers (168), who have examined this reaction in banana peel. Neumark (169) has shown the presence of tyrosine decarboxylase (L-tyrosine carboxy-lyase) in several different plants. There is, however, evidence to suggest that the second pathway may to some extent also be operative. Correale & Cortese (166), for example, showed that fresh suspensions of ground *Sarothamnus* seedlings could decarboxylate DOPA, and Andrews & Pridham (136) have also demonstrated weak decarboxylase (L-DOPA carboxy-lyase) activity in extracts of banana peel acetone powder which was incubated with pyridoxal-5′-phosphate and DOPA. It is interesting to note that DOPA decarboxylase is widespread in mammalian tissues and that it can be inhibited by hydroxycinnamic acid derivatives (170). Piccinelli (158), who has studied the amino acid and amine

TABLE I

HYDROXYPHENYLALKYLAMINES IN HIGHER PLANTS

Hydroxyphenylalkylamine	Examples of occurrence	Reference
Tyramine (β-(*p*-Hydroxyphenyl)ethylamine)	*Sarothamnus scoparius* *Hordeum vulgare*	(148) (149)
N-Methyltyramine	*Hordeum vulgare*	(149, 150)
Synephrine (β-(*p*-Hydroxyphenyl)-β-hydroxy-*N*-methyl-ethylamine)	*Citrus* spp.	(151)
Hordenine (β-(*p*-Hydroxyphenyl)-*N*,*N*-dimethylethyl-amine)	*Hordeum vulgare* *Anhalonium fissuratum*	(149) (152)
Candicine (Trimethyl-β-(*p*-hydroxyphenyl)ethyl ammonium cation)	*Trichocereus* spp. *Magnolia grandiflora*	(153, 154) (155)
DOPamine (β-(3,4-Dihydroxyphenyl)ethylamine)	*Musa sapientum* *Sarothamnus scoparius*	(156) (148, 157)
Epinine (β-(3,4-Dihydroxyphenyl)-*N*-methylethylamine)	*Sarothamnus scoparius* *Vicia faba*	(148) (158)
N-EthylDOPamine(?)	*Sarothamnus scoparius*	(159)
Noradrenaline (β-(3,4-Dihydroxyphenyl)-β-hydroxyethylamine)	*Portulaca oleracea* *Musa sapientum*	(160) (156)
Coryneine (Trimethyl-β-(3,4-dihydroxyphenyl)ethyl ammonium cation)	*Cereus coryne*	(161)
Salicifoline (Trimethyl-β-(3-hydroxy-4-methoxyphenyl) ethyl ammonium cation)	*Magnolia grandiflora*	(155)
Mescaline (β-(3,4,5-Trimethoxyphenyl)ethylamine)	*Anhalonium lewinii* *Trichocereus terschekii*	(162) (163)
N-Methylmescaline	*Anhalonium lewinii*	(164)
N-Acetylmescaline	*Anhalonium lewinii*	(165)
Trichocereine β-(3,4,5-Trimethoxyphenyl)-*N*,*N*-dimethylethylamine)	*Trichocereus terschekii*	(163)

content of *Vicia faba*, favors the second pathway for the formation of epinine in this plant.

The conversion of *N*-methyltyramine to epinine is also effected by *Sarothamnus* extracts (166), so the latter derivative could conceivably be formed by a modified version of the first pathway where *N*-methylation precedes hydroxylation. *N*-Methylation of ^{14}C-labelled tyramine by sprouting barley has been demonstrated by Leete, Kirkwood & Marion (171). It is obvious that a much more detailed examination of these systems is required before the relative importance of the various pathways can be ascertained.

With regard to the biosynthesis of the more complex amines in this group, Marion and his associates (171, 172), working with barley, have shown that the most likely pathway from tyrosine to hordenine is via tyramine and *N*-methyltyramine (see Fig. 4). The *N*-methyl groups in these reactions are

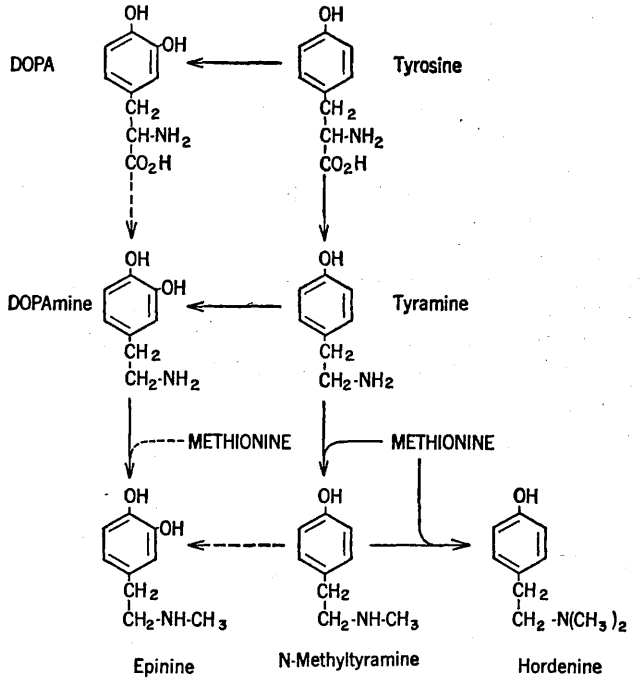


FIG. 4. The biosynthesis and metabolism of phenolic amino acids and amines. Arrows with broken lines indicate hypothetical or probable minor pathways.

probably derived from methionine (173). Tyrosine-2-^{14}C when fed to *Anhalonium lewinii* gives rise to mescaline labelled at C-1 (174). This conversion presumably involves a series of decarboxylation, hydroxylation, and methylation reactions, but the order in which these occur is not known. As with other groups of phenolic compounds, *o*-hydroxylation can probably be

catalysed by phenolase. For example, Correale & Cortese (166), again using their *Sarothamnus* seedling preparation, effected partial conversions of β-(*p*-hydroxyphenyl)-β-hydroxyethylamine to noradrenaline and candicine to coryneine.

The studies of Hasse & Schmid (175) suggest that quite a different pathway from those already discussed could be present in both higher and lower plants. These workers have shown that pea and lupin, for example, possess a transaminase which catalyses reactions between α-keto acids and aliphatic and aromatic amines with the formation of the corresponding aromatic aldehydes. The reaction is reversible and could, therefore, be utilized by the plant for amine formation.

Conclusion

There is now an understanding of at least some of the general reactions involved in the interconversions of monocyclic phenolic compounds in higher plants. It is very apparent that most of these compounds can be synthesized by a number of different pathways, and the relative importance of these may depend on the plant species, the age of the tissues, and the environmental conditions under which the plant is raised. The problems to be solved are, therefore, enormous. In particular there is a pressing need for detailed investigations of the enzymes which catalyse the individual steps in the reaction chains. One hopes that *in vitro* studies on coupled reactions involving several enzymes and their substrates will also be carried out and some insight of the metabolic control of these reactions be obtained. Finally, a knowledge of the relative distribution of enzymes and substrates in different tissues and in different parts of the cell would be extremely valuable and would, no doubt, make an important contribution to this field.

LITERATURE CITED

1. Bogorad, L., *Ann. Rev. Plant Physiol.*, **9**, 417 (1958)
2. Kremers, R. E., *Ann. Rev. Plant Physiol.*, **10**, 185 (1959)
3. Neish, A. C., *Ann. Rev. Plant Physiol.*, **11**, 55 (1960)
4. Brown, S. A., *Lloydia*, **26**, 211 (1963)
5. Brown, S. A., *Science*, **134**, 1 (1961)
6. Freudenberg, K., *Pure Appl. Chem.*, **5**, 9 (1962)
7. Neish, A. C., in *Formation of Wood in Forest Trees*, 219–39 (Zimmermann, M. H., Ed., Academic, New York, 1964)
8. Miller, L. P., *Contrib. Boyce Thompson Inst.*, **13**, 185 (1943)
9. Miller, L. P., *ibid.*, **11**, 271 (1940)
10. Macleod, N. J., and Pridham, J. B. (Unpublished)
11. Kennedy, J. S., and Mittler, T. E., *Nature*, **171**, 528 (1953); Mittler, T. E., *J. Exptl. Biol.*, **34**, 334 (1957); Mittler, T. E., *ibid.*, **35**, 74 (1958)
12. Bate-Smith, E. C., and Swain, T. (Unpublished)
13. Hathway, D. E., *Biochem. J.*, **71**, 533 (1959)
14. Hillis, W. E., and Carle, A., *Biochem. J.*, **74**, 607 (1960); Hillis, W. E., and Carle, A., *ibid.*, **82**, 435 (1962); Hillis, W. E., and Hasegawa, M., *Phytochemistry*, **2**, 195 (1963)
15. Wardrop, A. B., and Cronshaw, J., *Nature*, **193**, 90 (1962)
16. Bonner, J., *Am. J. Botany*, **36**, 429 (1949); Millerd, A., Bonner, J., and Biale, J. B., *Plant Physiol.*, **28**, 521 (1953); Stenlid, G., *Physiol. Plantarum*, **16**, 110 (1963)
17. Deyson, G., and Truhart, R., *Compt. Rend. Soc. Biol.*, **151**, 1678, 1719 (1957)
18. Frey-Wyssling, A., *Naturwissenschaften*, **33**, 500 (1942)
19. Whitehead, D. C., *Nature*, **202**, 417 (1964)
20. Pridham, J. B., in *Phenolics in Plants in Health and Disease*, 9–15 (Pridham, J. B., Ed., Pergamon, Oxford, 131 pp., 1960)
21. Asen, S., and Emsweller, S. L., *Phytochemistry*, **1**, 169 (1962)
22. Miwa, T., Nakamura, S., and Shibata, A., *Kôso Kagaku Shimpoziumu*, **12**, 48 (1957); *Chem. Abstr.*, **52**, 1314 (1958)
23. Hutchinson, A., Roy, C., and Towers, G. H. N., *Nature*, **181**, 841 (1958)
24. Pridham, J. B., and Saltmarsh, M. J., *Biochem. J.*, **87**, 218 (1963)
25. Pridham, J. B., *Phytochemistry*, **3**, 493 (1964)
26. Harborne, J. B., and Corner, J. J., *Biochem. J.*, **81**, 242 (1961)
27. Runeckles, K. C., and Woolrich, K., *Phytochemistry*, **2**, 1 (1963)
28. Pridham, J. B., *Advances in Carbohydrate Chemistry* (In press)
29. Cardini, C. E., and Leloir, L. F., *Ciencia Invest. (Buenos Aires)*, **13**, 514 (1957)
30. Pridham, J. B., and Saltmarsh, M. J., *Biochem. J.*, **74**, 42P (1960)
31. Yamaha, T., and Cardini, C. E., *Arch. Biochem. Biophys.*, **86**, 127 (1960)
32. Barber, G. A., *Biochemistry*, **1**, 463 (1962)
33. Trivelloni, J. C., Recondo, E., and Cardini, C. E., *Nature*, **195**, 1202 (1962)
34. Jacobelli, G., Tabone, M. J., and Tabone, D., *Bull. Soc. Chim. Biol.*, **40**, 955 (1958)
35. Pridham, J. B., *Anal. Chem.*, **28**, 1967 (1956)
36. Anderson, J. D., Hough, L., and Pridham, J. B., *Biochem. J.*, **77**, 564 (1960)
37. Cardini, C. E., and Yamaha, T., *Nature*, **182**, 1446 (1958); Cardini, C. E., and Yamaha, T., *Arch. Biochem. Biophys.*, **86**, 133 (1960)
38. Marsh, C. A., *Biochem. Biophys. Acta*, **44**, 359 (1960)
39. Marsh, C. A., *Nature*, **176**, 176 (1955); Pearl, I. A., and Darling, S. F., *J. Org. Chem.*, **28**, 1442 (1963)
40. Barber, G. A., *Biochem. Biophys. Res. Commun.*, **8**, 204 (1962)
41. Cabib, E., *Ann. Rev. Biochem.*, **32**, 321 (1963)
42. Pridham, J. B., *Biochem. J.*, **76**, 13 (1960)
43. Pridham, J. B., *Chem. Ind. (London)*, 1172 (1961)
44. Pridham, J. B., and Wallenfels, K., *Nature*, **202**, 488 (1964)
45. Nakamura, S., and Miwa, T., *Nature*, **202**, 291 (1964)
46. Harborne, J. B., *Phytochemistry*, **1**, 203 (1962)
47. Harborne, J. B., and Sherratt, H. S. A., *Biochem. J.*, **78**, 298 (1961)
48. Harborne, J. B., *Biochem. J.*, **84**, 100 (1962)
49. Harborne, J. B., *ibid.*, **74**, 262 (1960)
50. Baruah, P., and Swain, T., *J. Sci. Food Agr.*, **10**, 125 (1959)
51. Roberts, E. A. H., *Nature*, **185**, 536 (1960)

52. Haas, P., and Hill, T. G., *An Introduction to the Chemistry of Plant Products*, **I**, 243 (Longmans, Green & Co., New York, 401 pp., 1928)
53. Freudenberg, K., and Harkin, J. M., *Phytochemistry*, **2**, 189 (1963)
54. Furuya, M., Galston, A. W., and Stowe, B. B., *Nature*, **193**, 456 (1962)
55. Cardini, C. E., *Ciencia Invest. (Buenos Aires)*, **17**, 349 (1961)
56. Harrison, K., *Nature*, **181**, 1131 (1958)
57. Clark, V. M., Hutchinson, D. W., and Todd, A., *Nature*, **187**, 59 (1960)
58. Geissman, T. A., in *The Biosynthesis of Plant Products*, 563–616 (Bernfeld, P., Ed., Pergamon, Oxford, 930 pp., 1963)
59. Higuchi, T., and Kawamura, I., in *Modern Methods of Plant Analysis*, **VII**, 260–89 (Linskens, H. F., Sanwal, B. D., and Tracey, M. V., Eds., Springer, Heidelberg, 735 pp., 1964)
60. Levin, J. G., and Sprinson, D. B., *J. Biol. Chem.*, **239**, 1142 (1964)
61. Gibson, M. I., and Gibson, F., *Biochem. Biophys. Acta*, **65**, 160 (1962)
62. Gibson, F., and Jackman, M. L., *Nature*, **198**, 388 (1963)
63. Gamborg, O. L., and Simpson, F. J., *Can. J. Biochem.*, **42**, 583 (1964)
64. Karrer, W., *Konstitution und Vorkommen der organischen Pflanzenstaffe* (Birkhaeuser, Basel, 1207 pp., 1958)
65. Tomaszewski, M., *Bull. Acad. Polon. Sci. Ser. Sci. Biol.*, **8**, 61 (1960)
66. Hattori, S., and Sâto, M., *Phytochemistry*, **2**, 385 (1963)
67. Dupy, P., and Puisais, J., *Compt. Rend.*, **241**, 48 (1955)
68. Madhusudanan Nair, P., and Vaidyanathan, C. S., *Phytochemistry*, **3**, 235 (1964)
69. Madhusudanan Nair, P., and Vaidyanathan, C. S., *ibid.*, 513
70. Stainer, R. Y., and Hayaishi, O., *J. Bacteriol.*, **62**, 367, 690 (1951)
71. Cartwright, R. A., Roberts, E. A. H., Flood, A. E., and Williams, A. H., *Chem. Ind. (London)*, 1062 (1955)
72. Goldschmidt, O., and Hergert, H. L., *Tappi*, **44**, 858 (1961); Maier, V. P., Metzler, D. M., and Huber, A. F., *Biochem. Biophys. Res. Commun.*, **14**, 124 (1964)
73. Corner, J. J., Harborne, J. B., Humphries, S. G., and Ollis, W. D., *Phytochemistry*, **1**, 73 (1962)
74. El-Basyouni, S. Z., and Towers, G. H. N., *Can. J. Biochem.*, **42**, 493 (1964)
75. Hiramoto, M., and Watanabe, K., *J. Pharm. Soc. Japan*, **59**, 261 (1939); *Chem. Abstr.*, **34**, 1006 (1940)
75a. Bate-Smith, E. C., *J. Linn. Soc. (Botan.)*, **58**, 95 (1962)
76. Koukol, J., and Conn, E. E., *J. Biol. Chem.*, **236**, 2692 (1961)
77. Neish, A. C., *Phytochemistry*, **1**, 1 (1961)
78. Brown, S. A., *Can. J. Botany*, **39**, 253 (1961)
79. Macleod, N. J., and Pridham, J. B., *Biochem. J.*, **88**, 45P (1963)
80. Brown, S. A., Wright, D., and Neish, A. C., *Can. J. Biochem. Physiol.*, **37**, 25 (1959)
81. Gamborg, O. L., Wetter, L. R., and Neish, A. C., *Phytochemistry*, **1**, 159 (1962)
82. Gamborg, O. L., and Wetter, L. R., *Can. J. Biochem. Physiol.*, **41**, 1733 (1963)
83. Uspenskaya, J. V., Kretovich, W. L., and Kashkarayeva, K. I., *Biokhimiya*, **28**, 1025 (1963)
84. Higuchi, T., and Brown, S. A., *Can. J. Biochem. Physiol.*, **41**, 621 (1963)
85. McCalla, D. R., and Neish, A. C., *Can. J. Biochem. Physiol.*, **37**, 537 (1959)
86. Kosuge, T., and Conn, E. E., *J. Biol. Chem.*, **234**, 2133 (1959)
87. Grisebach, H., and Vollmer, K. O., *Z. Naturforsch.*, **18**(b), 753 (1963)
88. El-Basyouni, S. Z., Chen, D., Ibrahim, R. K., Neish, A. C., and Towers, G. H. N., *Phytochemistry*, **3**, 485 (1964)
89. Levy, C. C., and Zucker, M., *J. Biol. Chem.*, **235**, 2418 (1960)
90. Reznik, H., and Urban, R. U., *Naturwissenschaften*, **44**, 592 (1957)
91. Kratzl, K., *Tappi*, **43**, 650 (1960)
92. Higuchi, T., and Brown, S. A., *Can. J. Biochem. Physiol.*, **41**, 613 (1963)
93. Zenk, M. H., *Z. Naturforsch*, **19**(b), 83 (1964)
94. Higuchi, T., and Brown, S. A. (Unpublished, reported in ref. 84)
95. Finkle, B. J., Lewis, J. C., Corse, J. W., and Lundin, R. E., *J. Biol. Chem.*, **237**, 2926 (1962)
96. Hattori, S., and Imaseki, H., *J. Am. Chem. Soc.*, **81**, 4424 (1959)
97. Brown, S. A., Towers, G. H. N., and Chen, D., *Phytochemistry*, **3**, 649 (1964)
98. Brown, S. A., Towers, G. H. N., and Wright, D., *Can. J. Biochem. Biophys.*, **38**, 143 (1960)

99. Kosuge, T., and Conn, E. E., *J. Biol. Chem.*, **236**, 1617 (1961)
100. Stoker, J. R., and Bellis, J. M., *J. Biol. Chem.*, **237**, 2303 (1962)
101. Stoker, J. R., *Biochem. Biophys. Res. Commun.*, **14**, 17 (1960)
102. Sondheimer, E., and Griffin, D. H., *Science*, **131**, 672 (1960)
103. Rabin, R. S., and Klein, R. M., *Arch. Biochem. Biophys.*, **70**, 11 (1957)
104. Gortner, W. A., Kent, M. J., and Sutherland, G. A., *Nature*, **181**, 630 (1958)
105. Varga, M., and Koves, E., *Naturwissenschaften*, **49**, 355 (1962)
106. Mumford, F. E., Stark, H. M., and Smith, D. H., *Phytochemistry*, **2**, 215 (1963)
107. Gamborg, O. L., Wetter, L. R., and Neish, A. C., *Can. J. Biochem. Physiol.*, **39**, 1113 (1961)
108. Schwimmer, S., *J. Biol. Chem.*, **232**, 715 (1958)
109. Griffiths, L. A., *Nature*, **182**, 733 (1958)
110. Koves, E., and Varga, M., *Phyton (Buenos Aires)*, **12**, 93 (1959)
111. Ibrahim, R. K., and Towers, G. H. N., *Nature*, **184**, 1803 (1959)
112. Ibrahim, R. K., and Towers, G. H. N., *Arch. Biochem. Biophys*,. **87**, 125 (1960)
113. Bohm, B. A., and Towers, G. H. N., *Can. J. Botany*, **40**, 677 (1962)
114. Ibrahim, R. K., Towers, G. H. N., and Gibbs, R. D., *J. Linn. Soc. (Botan.)*, **58**, 223 (1962)
115. Gross, S. R., *J. Biol. Chem.*, **233**, 1146 (1958)
116. Albrecht, H., and Bernhard, K., *Helv. Chim. Acta*, **30**, 627 (1947)
117. Haslam, E., Haworth, R. D., and Knowles, P. F., *J. Chem. Soc.*, 1854 (1961)
118. Bassett, E. W., and Tanenbaum, S. W., *Biochem. Biophys. Acta*, **28**, 247 (1958)
119. Gibson, M. I., and Gibson, F. (Unpublished, reported in ref. 62)
120. Conn, E. E., and Swain, T., *Chem. Ind. (London)*, 592 (1961)
121. Geissman, T. A., and Hinreiner, E., *Botan. Rev.*, **18**, 165 (1952)
122. Henderson, M. E. K., and Farmer, V. C., *J. Gen. Microbiol.*, **12**, 37 (1955)
123. Gross, S. R., and Schütte, H. R., *Arch. Pharm.*, **296**, 1 (1963)
124. Ohle, H., *Biochem. Z.*, **131**, 611 (1922)
125. Zenk, M. H., and Müller, G., *Z. Naturforsch.*, **19**(b), 398 (1964)
126. Klämbt, H. D., *Nature*, **196**, 491 (1962)
127. Byrrum, R. V., Flokstra, T. H., Dewey, L. J., and Ball, C. D., *J. Biol. Chem.*, **210**, 633 (1954)
128. Finkle, B. J., and Nelson, R. F., *Biochem. Biophys. Acta*, **78**, 747 (1963)
129. Finkle, B. J., and Masri, M. S., *Biochim. Biophys. Acta*, **85**, 167 (1964)
130. Buhler, D. R., and Mason, H. S., *Arch. Biochem. Biophys.*, **92**, 424 (1961)
131. Pridham, J. B., and Young, M., *Biochem. J.*, **92**, 20P (1954)
132. Bachman, D. M., Dragoon, B., and John, S., *Arch. Biochem. Biophys.*, **91**, 326 (1960)
133. Bruns, F. H., and Fiedler, L., *Nature*, **181**, 1533 (1958)
134. Gander, J. E., *Federation Proc.*, **18**, 232 (1959)
135. Guggenheim, M., *Z. Physiol. Chem.*, **88**, 276 (1913)
136. Andrews, R. S., and Pridham, J. B. (Unpublished results)
137. Miller, E. R., *J. Biol. Chem.*, **44**, 481 (1920)
138. Pilarczyk, W., *Planta Med.*, **6**, 258 (1958)
139. Watanabe, T., *Nippon Sanshigaku Zasshi*, **25**, 439 (1956)
140. Liss, I., *Naturwissenschaften*, **43**, 304 (1961)
141. Nagasawa, T., Takagi, H., Kawakami, K., Suzuki, T., and Sahashi, Y., *Agr. Biol. Chem. (Tokyo)*, **25**, 441 (1961)
142. Andrews, R. S., and Pridham, J. B., *Nature* (In press)
143. Evans, W. C., and Raper, H. S., *Biochem. J.*, **31**, 2155 (1937)
144. Liss, I., *Flora*, **151**, 35 (1961)
145. Kenten, R. H., *Biochem. J.*, **67**, 300 (1957); **68**, 244 (1958)
146. Goldschmiedt, G., *Monatsh. Chem.*, **33**, 1379 (1912); **34**, 659 (1913)
147. Kjaer, A., and Larsen, P. O., *Acta Chem. Scand.*, **17**, 2397 (1963)
148. Correale, P., and Cortese, I., *Naturwissenschaften*, **40**, 57 (1953)
149. Erspamer, V., and Falconieri, G., *Naturwissenschaften*, **39**, 431 (1952)
150. Kirkwood, S., and Marion, L., *J. Am. Chem. Soc.*, **72**, 2522 (1950)
151. Newhall, W. F., and Edwards, G. J., *J. Biol. Chem.*, **239**, 930 (1964)
152. Hefter, A., *Chem. Ber.*, **27**, 2975 (1894)
153. Reti, L., *Compt. Rend. Soc. Biol.*, **114**, 811 (1933)
154. Reti, L., and Arnolt, R. I., *Actas Trabajos Congr. Med. Roasario*, **50**, **3**, 39 (1935)

Phytochemistry, 1966, Vol. 5, pp. 777 to 781. Pergamon Press Ltd. Printed in England

OBSERVATIONS ON THE TRANSLOCATION OF PHENOLIC COMPOUNDS*

NORMA J. MACLEOD and J. B. PRIDHAM

Department of Chemistry, Royal Holloway College (University of London), Englefield Green, Surrey

(*Received* 20 *December* 1965)

Abstract—The rates of translocation of phenolic compounds introduced into the apical leaves of *Vicia faba* have been estimated. Some evidence for the occurrence of phenolic compounds in the sieve tubes of *Salix* and *Vicia* has been obtained using "aphid techniques".

INTRODUCTION

THERE is now a substantial amount of information regarding the biosynthetic pathways involved in the formation of phenolic compounds in plants.[1] Precursors such as acetate and the cinnamic acids can be produced in photosynthesizing tissues and there be further elaborated to flavonoids and higher molecular weight phenolics. Whether phenolic compounds can actually move through the tissues and, in particular, be translocated in the phloem is debatable at the present time. In the case of woody plants, it would be interesting to know whether the flavonoids, tannins and lignins found in the bark and wood arise from phenolic compounds formed in the leaves or from more common metabolites such as acetate and/or carbohydrates.

Hillis and his associates[2–4] favour the theory that the formation of *Eucalyptus* wood polyphenolics occurs *in situ* from carbohydrates. They demonstrated the translocation of ^{14}C-labelled D-glucose from the phloem to the heartwood–sapwood boundary followed by the incorporation of label into the phenolics. The histological studies of Wardrop and Cronshaw[5] using *Eucalyptus* also suggested that the tannins were produced from carbohydrate; in this case starch. Hathway[6] on the basis of ringing experiments with *Quercus pedunculata* claimed that (+)-gallocatechin and leucodelphinidin were translocated down from the leaves to the trunk and there oxidized to phlobotannins. In the case of phenolic glycosides, Miller[7] believed that they accumulated at sites of low metabolic activity and remained in these tissues until the death of the plant. His conclusions were mainly based on the treatment of gladiolus corms with *o*-chlorophenol which was converted to the β-gentiobioside; this was not translocated to the shoots when these corms were germinated or to

* This work was supported by grants from the Agricultural Research Council and the United States Department of Agriculture.

[1] J. B. HARBORNE (Ed.) *Biochemistry of Phenolic Compounds*. Academic Press, New York (1964).
[2] W. E. HILLIS and A. CARLE, *Biochem. J.* **74**, 607 (1960).
[3] W. E. HILLIS and A. CARLE, *Biochem. J.* **82**, 435 (1963).
[4] W. E. HILLIS and M. HASEGAWA, *Phytochem.* **2**, 195 (1963).
[5] A. B. WARDROP and J. CRONSHAW, *Nature* **193**, 90 (1962).
[6] D. E. HATHWAY, *Biochem. J.* **71**, 533 (1959).
[7] L. P. MILLER, *Contrib. Boyce Thompson Inst.* **11**, 271 (1940).

resulting daughter corms. Macleod and Pridham[8] were unable to confirm this using quinol-treated *Vicia faba* seeds. The young shoots from the germinating seeds did contain arbutin. No movement of natural flavonoids from scions to stocks could be detected when Delavean[9] examined grafts of *Tropaeolum* spp. Gorz and Haskins,[10] however, were able to show that small amounts of coumarin could be translocated across graft unions.

The experiments we now wish to describe support the idea that phenolic compounds can and are translocated in the phloem of higher plants.

RESULTS AND DISCUSSION

Initial experiments with *Vicia faba* plants (~30 cm high) showed that resorcinol, when introduced into the main vein of an apical leaf, became distributed over the whole plant, including the roots, within 30 min. A similar, although somewhat slower distribution, was effected by placing small volumes of resorcinol and other phenols dissolved in a dilute solution of "Tween 40" on the epidermal surfaces of the laminae. The minimum rates of movement of a number of foreign and naturally occurring phenols, introduced into the vein, are given in Table 1.

TABLE 1. PHENOL TRANSLOCATION RATES IN *Vicia faba*

Phenol	Rate of movement down stem (cm/hr)
m-Hydroxyphenyl-β-D-glucoside	108
Arbutin	90
Aesculin	84
Salicin	66
Resorcinol	60
Catechol	54
Phloroglucinol	54
Quinol	48
Saligenin	42
Caffeic acid	42
Ferulic acid	30
Kaempferol	18
Quercetin	12

The general translocation rates for phenols (with the exception of the flavonoids) appear to fall within the range found by others for ^{14}C-assimilates (see Kursanov[11]). There is also fairly good evidence that glycosidic derivatives migrate more rapidly than the phenolic aglycones. The low rates observed with the flavonoids may be due to poor penetration and/or insolubility factors. Roberts[12] has suggested that the planar structure of these compounds may adversely affect their translocation. When resorcinol, catechol, quinol and saligenin were fed the corresponding mono β-D-glucosides were also observed in the tissues.

Although these results show the feasibility of phenol translocation they can be criticized on the grounds that the compounds tested were foreign to the plant and/or, were introduced into the phloem in a "non-physiological" manner. The only certain way of showing that

[8] N. J. MACLEOD and J. B. PRIDHAM. Unpublished results.
[9] P. G. DELAVEAN, *Compt. Rend.* **258**, 318 (1964).
[10] H. J. GORZ and F. A. HASKINS, *Crop Sci.* **2**, 255 (1962).
[11] A. L. KURSANOV, *Advanc. Botan. Res.* **1**, 209 (1963).
[12] E. A. H. ROBERTS, *Nature* **185**, 536 (1960).

phenols are translocated is to demonstrate their natural occurrence as sieve-tube constituents. This can be achieved with little risk of contamination from phenols in surrounding tissues by the "aphid stylet" technique using *Tuberolachnus salignus*.[13] Like other aphids, this organism feeds directly from sieve-tubes. Our own attempts to use this procedure with *Salix* spp. were unsuccessful although Bate-Smith and Swain[14] report that phloem exudates obtained in this way from similar species did contain small amounts of fluorescent materials which appeared to be phenolic on paper chromatograms and which gave u.v. spectra characteristic of cinnamic acid derivatives.

T. salignus is a large aphid and therefore ideally suited for the "aphid-stylet" technique. Aphids feeding on herbaceous hosts are usually smaller and more delicate and hence cannot readily be dissected whilst feeding. The phloem constituents can, however, be examined by an analysis of the gut contents or the honeydew but this method does suffer from the disadvantage that the enzymes in the alimentary tract (cf. Auclair[15]) may modify the constituents as they pass through.

Therefore, before utilizing *Macrosiphum pisi* for an examination of the phloem of *Vicia faba*, buffered extracts of the aphid were examined for enzyme activity. Thus α- and β-D-glucoside:glucohydrolases, α-D-galactoside:galactohydrolase and an esterase capable of hydrolysing chlorogenic acid were all shown to be present (Table 2). The location of these

TABLE 2. ENZYME ACTIVITY IN *M. pisi* EXTRACTS

Substrate	Products	Inference
Maltose	Glucose	α-D-Glucoside:glucohydrolase
Isomaltose	Glucose	
Methyl-α-D-glucoside	Glucose	
Raffinose	Galactose, sucrose	α-D-Galactoside:galactohydrolase
Cellobiose	—	
Methyl-β-D-glucoside	Glucose	β-D-Glucoside:glucohydrolase
Gentiobiose	Glucose	
Arbutin	Glucose, quinol	
Salicin	Glucose, saligenin	
Chlorogenic acid	Caffeic acid, quinic acid	Esterase
Catechol	"Melanin"	Phenolase
(+)-Catechin	"Melanin"	

enzymes in the body was not determined but it is reasonable to assume that some, at least, must have been present in the alimentary tract. Phenolase activity was also apparent in the *M. pisi* extracts. This enzyme complex is common in insects but here again little is known about its location except that it occurs in some bloods and cuticles.[16] The enzymic modification of dietary phenolics was therefore considered to be a possibility but it seemed unlikely that this would be a rapid process and, more important, that the actual synthesis of phenols from non-phenolic compounds would occur.

An examination of the gut contents of *M. pisi* feeding on *V. faba* stems after introduction of foreign phenols into the apical leaves confirmed that there was little change in the compounds that were fed. Thus quinol, catechol, resorcinol, ferulic acid, caffeic acid and arbutin

[13] J. S. KENNEDY and T. E. MITTLER, *Nature* **171**, 528 (1953).
[14] E. C. BATE-SMITH and T. SWAIN. Unpublished results.
[15] J. L. AUCLAIR, *Ann. Rev. Entomol.* **8**, 439 (1963).
[16] C. B. COTTRELL, *Advances Insect Physiol.* **2**, 175 (1964).

were all present after 1 hr in aphids feeding at stem sites approximately 10–15 cm from the points of introduction of the phenols. Some slight hydrolysis of arbutin to quinol had occurred either in the plant or the alimentary tract and no glucosides appeared to have been synthesized. This suggests that the glucosylation observed in the earlier experiments did not occur in the sieve-tubes but in the adjacent tissues. The honeydew from *M. pisi* feeding on the stems of untreated *V. faba* plants contained β-(3,4-dihydroxy-phenyl)-L-alanine (DOPA), which occurs in relatively high concentrations in this plant (Guggenheim[17]; Andrews and Pridham[18]), and hence appears to be translocatory material. Other compounds giving azo dyes with diazotized *p*-nitroaniline/NaOH were also detected as sieve-tube components.

Paper chromatographic analysis of the honeydew from *T. salignus* feeding on branches of *Salix daphnoides* showed the presence of compounds with phenolic properties which were also detected in leaf and bark extracts of the plant (Table 3). One compound was positively identified as tyrosine.

TABLE 3. SOME COMPONENTS FOUND IN *T. salignus* HONEYDEW AND THE BARK (B) AND LEAVES (L) OF *S. daphnoides*

R_f values in EAW solvent			
B and L components	Honeydew components	Properties	Identity
0·34 (B, L)	0·34	u.v., blue fluorescent	Cinnamic derivative?
0·59 (B)	0·59	Diazo spray, red Ninhydrin, blue	Tyrosine
0·72 (L)	0·72	u.v., blue fluorescent	Cinnamic derivative?
0·92 (B)	0·92	u.v. blue fluorescent Diazo spray, red	

The results of this study show clearly that phenols introduced into the leaves can move down the plant by the normal processes of translocation and there is definite evidence for the occurrence of phenolic compounds as natural constituents of the sieve-tubes. The presence of tyrosine in the sieve-tubes of *Salix* has been clearly established and this phenolic amino acid has been noted, together with phenylalanine, in honeydews from aphids feeding on a number of different hosts.[15] On the basis of this observation alone there is no reason to suppose that all complex phenolic compounds are biosynthesized *in situ* from non-aromatic derivatives. These two amino acids could be translocated to tissues possessing the necessary ammonia lyases[19, 20] and there be converted to polyphenolics via the corresponding cinnamic acids. The presence of DOPA in the sieve-tubes of *V. faba* is interesting in view of its probable metabolic importance in other plants in alkaloid,[21] betacyanin[22, 23] and, possibly, caffeic acid[24] formation.

[17] M. GUGGENHEIM, *Z. Physiol. Chem.* **88**, 276 (1913).
[18] R. S. ANDREWS and J. B. PRIDHAM. Unpublished results.
[19] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[20] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[21] E. RAMSTAD and S. AGURELL, *Ann. Rev. Plant Physiol.* **15**, 143 (1964).
[22] L. HÖRHAMMER, H. WAGNER and W. FRITZSCHE, *Biochem. Z.* **339**, 398 (1964).
[23] L. MINALE, M. PIATTELLI and R. A. NICOLAUS, *Phytochem.* **4**, 593 (1965).
[24] N. J. MACLEOD and J. B. PRIDHAM, *Biochem. J.* **88**, 45P (1963).

EXPERIMENTAL

Vicia faba (Var Johnson's Longpod) plants, approximately 30 cm high, were used in all the feeding and aphid experiments which were carried out at room temperature. Small branches of *Salix daphnoides* infected with *Tuberolachnus salignus* were obtained from the grounds of Royal Holloway College.

Paper Chromatography

Phenolic compounds were examined on Whatman No. 3 paper using *n*-butanol–ethanol–water (40:11:19; BEW) and ethylacetate–acetic acid–water (9:2:2; EAW) solvents. Location was effected by u.v. light, diazotized *p*-nitroaniline/NaOH and, in the case of phenolic amino acids, ninhydrin. EAW solvent was used for sugars and quinic acid which were located with *p*-anisidine hydrochloride and $AgNO_3$–NaOH, respectively.

Phenol Translocation Rate

Aqueous solutions (1%, w/v) of phenols were fed into apical leaves of *V. faba* plants for periods varying from 10 min to 3 hr. This was achieved by partially dissecting the main veins from the laminae, dipping these veins into the solutions contained in 5-ml beakers and then cutting off the ends of the submerged veins. After treatment, the stems and roots of the plants were quickly cut into 1 cm sections and these were extracted with aqueous methanol (80%, v/v) and examined on paper chromatograms. The rate of translocation in cm/hr could thus be calculated.

Enzyme Activity in Macrosiphum pisi *Extracts*

Extracts were prepared by macerating feeding aphids with 0·05 M-sodium acetate buffer (pH 5·6; 5 aphids/2 ml buffer) and incubating (25°) the centrifuged preparations with various substrates at 0·5% (w/v) concentrations. The products were examined on chromatograms (see Table 2). Control reaction mixtures using boiled enzyme preparations were also examined.

Location of Phenols Fed to V. faba *Using* M. pisi

All but the apical leaves were removed from *V. faba* plants and aphids then placed on the main stems 10–15 cm down from the leaves. The organisms were confined to these regions by cotton wool packed around the stems. Phenols were introduced into the veins, as previously described, and after 1 hr the aphids removed and aqueous extracts examined on paper chromatograms.

Collection of Honeydew

This was achieved by allowing it to fall on to glass plates placed beneath the plants. The individual globules of syrup were then dissolved in small volumes of water and taken up in capillaries for chromatographic analysis. In other cases aphids were induced to excrete honeydew by anal stimulation with a needle. The droplets could then be collected directly with capillary tubes.

Extraction of Plant Tissues

This was effected with cold aqueous methanol (80%, v/v). The solutions were filtered and concentrated in a rotary evaporator at 40°.

Phytochemistry, 1967, Vol. 6, pp. 13 to 18. Pergamon Press Ltd. Printed in England

MELANINS FROM DOPA-CONTAINING PLANTS

R. S. ANDREWS* and J. B. PRIDHAM

Department of Chemistry, Royal Holloway College, University of London, Englefield Green, Surrey

(*Received* 1 *June* 1966)

Abstract—The melanins from plants which contain DOPA and related compounds have been examined and shown to be largely composed of the catechol-type pigment. Some indole units also appear to be present, however.

INTRODUCTION

NICOLAUS, Piattelli and associates have shown that animal melanin pigments have structures based on indole and they suggest that catechol melanins may predominate in the higher plants and some fungi.[1] The type of melanin present in a particular organism can be ascertained by procedures such as alkaline fusion and nitrogen determinations. The latter give relatively high values (*ca.* 6–7%) for animal melanins and low (*ca.* 1%) for the plant pigments. Typical animal melanins yield 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid when fused with alkali. These indole derivatives were not produced from a number of higher plant and fungal melanins.[2] Instead the degradations yielded simple aromatic compounds such as catechol, protocatechuic acid and salicylic acid.

Only a few plant species appear to contain β-(3,4-dihydroxyphenyl)-L-alanine (DOPA) as a natural constituent, and many of these exhibit blackening (melanogenesis) reactions. For example, the broad-bean (*Vicia faba*) has relatively high concentrations of DOPA in many organs[3,4] and produces a black pigment in the flowers, the senile leaves and pods and the seed hila. Similarly, DOPA is found in *V. angustifolia*, *Astragalus cicer*, *Lupinus polyphyllus* and *Baptisia australis*,[4] and these plants exhibit blackening. Melanin pigmentation also occurs in species which contain compounds related to DOPA. The banana (*Musa* sp.), which has β-(3,4-dihydroxyphenyl) ethylamine (dopamine) as a constituent, is an example of this.[5]

It has commonly been assumed that plants containing DOPA and related derivatives produce melanins by biochemical pathways similar, but not identical to those outlined by Evans and Raper in 1937[6] (e.g. Thomson;[7] Pridham[8]). It was of interest, therefore, to examine melanins from DOPA-containing species in the light of the more recent discoveries by Nicolaus *et al.*[1,2]

* Present address: Department of Surgery, Royal Infirmary of Sheffield, Sheffield 6.

[1] R. A. NICOLAUS and M. PIATTELLI, *Rend. Accad. Sci. Fis. Mat.* **32**, 3 (1965).
[2] M. PIATTELLI, R. A. NICOLAUS and E. FATTORUSSO, *Tetrahedron* **20**, 1163 (1964).
[3] M. GUGGENHEIM, *Z. physiol. Chem.* **88**, 276 (1913).
[4] R. S. ANDREWS and J. B. PRIDHAM, Unpublished results.
[5] L. A. GRIFFITHS, *Nature* **184**, 58 (1959).
[6] W. C. EVANS and H. S. RAPER, *Biochem. J.* **31**, 2162 (1937).
[7] R. H. THOMSON, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 351. Academic Press, New York (1965).
[8] J. B. PRIDHAM, *Ann. Rev. Plant Physiol.* **16**, 13 (1965).

RESULTS AND DISCUSSION

Melanins were isolated after exhaustive solvent extraction and acid degradation of tissues from the pods and flowers of *V. faba*, the pods of *V. angustifolia*, *A. cicer*, *L. polyphyllus* and *B. australis* and banana skins. In addition, synthetic melanins were prepared by the oxidation of L-tyrosine and catechol with a potato phenolase preparation. All of these pigments gave very similar i.r. spectra (Figs. 1–4) which closely resembled those published by Bonner and Duncan for various animal melanins.[9] Little structural information can be gained by i.r. measurements with those materials but absorption bands at 1670–1690 cm^{-1} (strong) and 1250 cm^{-1} (broad) which are normally assigned to carboxyl groups[10] were

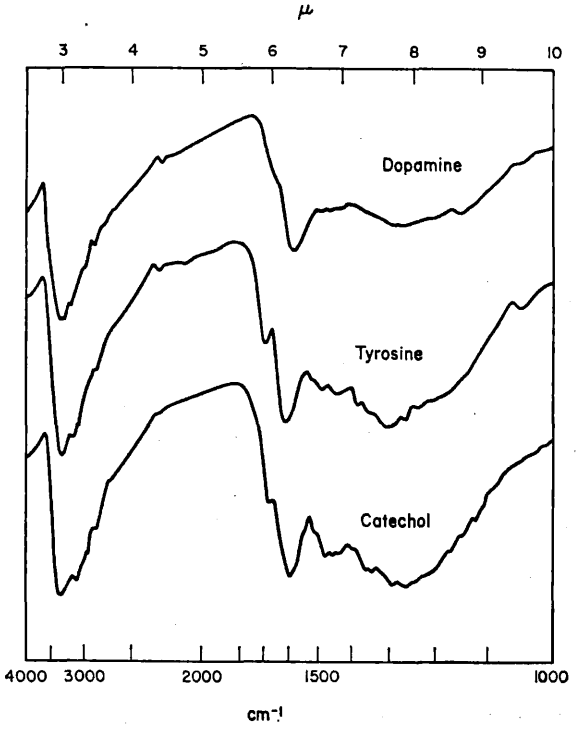


FIG. 1. INFRA-RED SPECTRA OF MELANINS PREPARED BY THE OXIDATION OF DOPAMINE, TYROSINE AND CATECHOL WITH POTATO PHENOLASE.

observed with most of the natural pigments. In the case of the synthetic melanins (Fig. 1) from DOPA and catechol the 1670–1690 cm^{-1} bands were weak and with the dopamine melanin, negligible. It is interesting to note that the banana skin melanin (Fig. 2) also showed negligible absorption at 1670–1690 cm^{-1}. Treatment of the pigments from *V. faba* (flowers), *V. angustifolia*, *A. cicer* and *B. australis* (Figs. 3 and 4) with alkali resulted in a marked reduction of the characteristic carboxyl bands and their replacement by a weak band at 1380 cm^{-1}, presumably due to ionization. One further strong band at 1600 cm^{-1} was exhibited by all the natural and synthetic melanins. Simple quinonoid structures normally give bands in the

[9] T. G. BONNER and A. DUNCAN, *Nature* **194**, 1078 (1962).
[10] L. J. BELLAMY, *The Infrared Spectra of Complex Molecules*, 2nd. Edn., Methuen, London (1962).

region 1645–1680 cm^{-1} [11] but a lowering of the frequency to 1600 cm^{-1} occur in the extended and possibly chelated quinones.

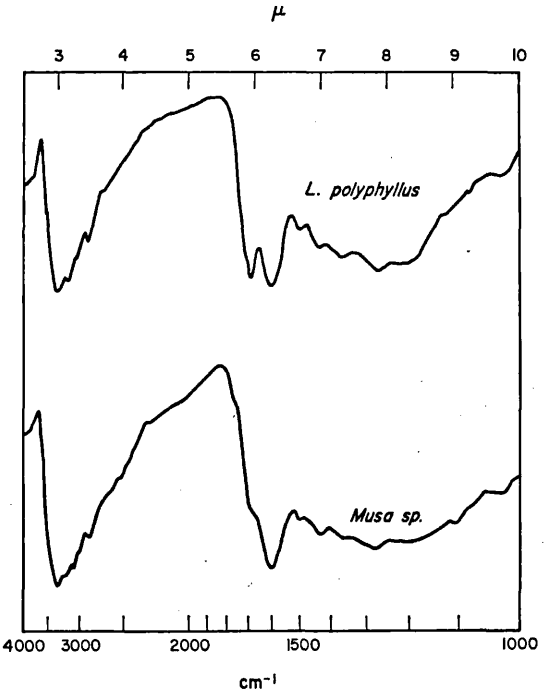


FIG. 2. INFRA-RED SPECTRA OF MELANINS FROM *L. polyphyllus* PODS AND BANANA (*Musa sp.*) SKINS.

TABLE 1. NITROGEN CONTENT AND ALKALINE FUSION PRODUCTS OF MELANINS

Source	%N	Alkaline fusion products detected
Astralagus cicer pods	1·2	—
Baptisia australis pods	1·6	—
Lupinus polyphyllus pods	1·3	Catechol, protocatechuic acid 5,6-dihydroxyindole.
Musa sp. fruit epicarp.	1·5	—
Vicia angustifolia pods	1·4	Catechol, protocatechuic acid 5,6-dihydroxyindole.
Vicia faba		
Flowers	2·1	Catechol, protocatechuic acid 5,6-dihydroxyindole.
pods	1·3	—
Tyrosine (synthesized with phenolase)	7·1	—
Dopamine (synthesized with phenolase)	6·8	—

The nitrogen contents of the pigments are given in Table 1. The low values obtained with the Papillionaceous melanins suggest that they are all of the catechol type. In the case of the

[11] R. N. JONES and C. SANDORFY, In *Chemical Applications of Spectroscopy* (Edited by W. WEST) Vol. 9, p. 274. Interscience, New York (1956).

polymers from *L. polyphyllus*, *V. faba* (flowers) and *V. angustifolia* this was further confirmed by alkaline fusion (Table 1) which yielded catechol, and protocatechuic acid. Small amounts of 5,6-dihydroxyindole, presumably from an indole melanin, were, however, definitely present in the fusion products. It can always, of course, be argued that oxidative polymerization of DOPA to a melanin was an artifact occurring perhaps during the isolation of pigment from the tissues. Although this is possible, it is reasonable to assume that this reaction does occur to some extent *in vivo* (particularly in organs such as *V. faba* flowers which contain

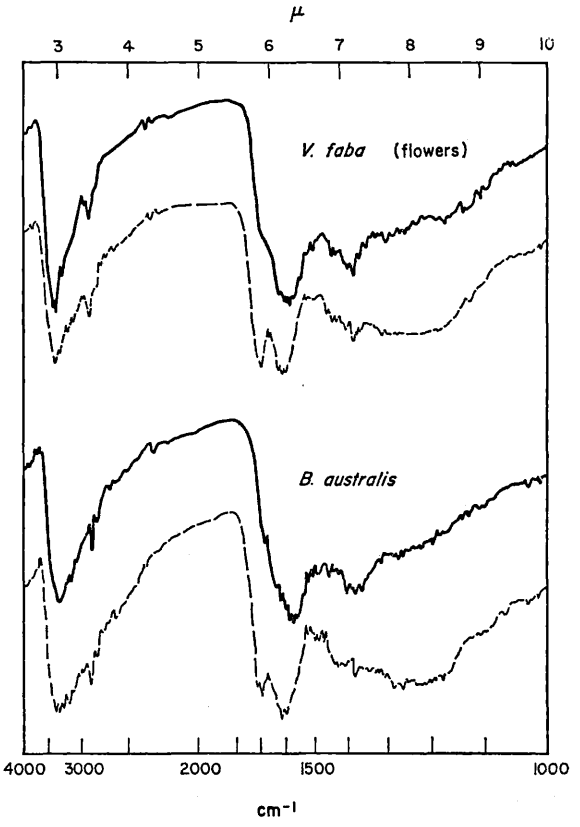


FIG. 3. INFRA-RED SPECTRA OF MELANINS FROM *V. faba* FLOWERS AND *B. australis* PODS.
———=after alkali treatment; — — —=normal spectrum.

ca. 11 mg DOPA/g F.W.[4]) producing a pigment largely composed of catechol residues co-polymerized with some indole units. The slightly elevated nitrogen content (2·1%) of the bean flower melanin is perhaps significant. What is most remarkable is the fact that more DOPA is not incorporated into the tissue melanins by a pathway approximating to that envisaged by Evans and Raper.[6] Piattelli and his associates believe that catechol melanins are fomed *in vivo* by oxidative polymerization of catechol itself. Some support for this idea came from the isolation of catechol from an alcoholic extract of the spores of *Ustilago maydis*: these contain a catechol melanin.[12] In higher plants catechol does not appear to be a common

[12] M. PIATTELLI, E. FATTORUSSO, R. A. NICOLAUS and S. MAGNO, *Tetrahedron* **21**, 3229 (1965).

constituent,[13, 14] but it has been shown that anthranilic acid, a derivative of the shikimic acid pathway, can be converted to catechol by a chloroplastic enzyme system from *Tecoma stans*.[15]

Nagasawa *et al.*[16] investigated the browning of broad-bean seed testas and suggested that this pigment arose from a DOPA-glucoside (cf. Andrews and Pridham[17]) which was first hydrolysed and then oxidized by phenolase. However, there is at present no evidence to confirm that the brown pigment is derived from DOPA. Our attempts to obtain the pigment from the mature testas by digestion with strong HCl resulted in the formation of antho-

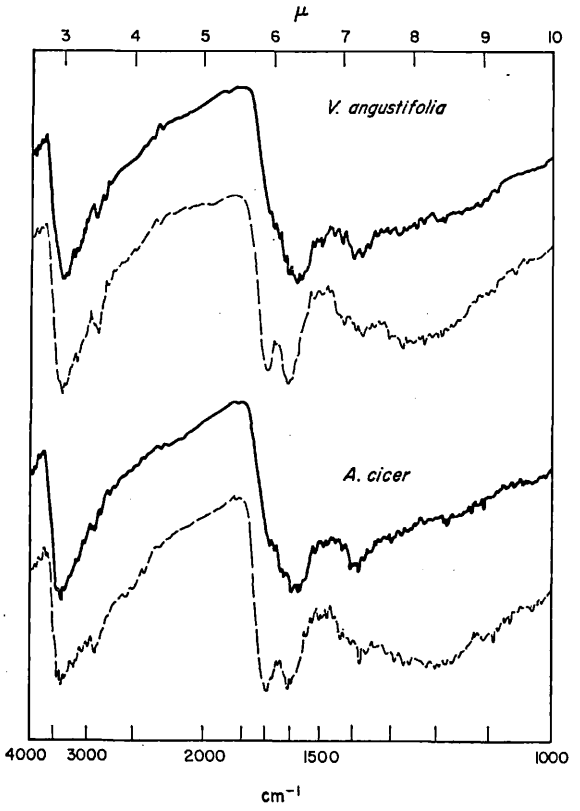


FIG. 4. INFRA-RED SPECTRA OF MELANINS FROM *V. angustifolia* AND *A. cicer* PODS.
———=after alkali treatment; — — —=normal spectrum.

cyanidins followed by a rapid blackening of the tissues even when the reaction was carried out under nitrogen. The final product, a very dark brown powder, had a low nitrogen content (1·1%) and on alkaline fusion it yielded phloroglucinol as a major product together with other unidentified phenolic compounds. These results suggest that the product was

[13] W. KARRER, *Konstitution und Vorkommen der organischen Pflanzenstoffe*, Birkhauser, Basle (1958).
[14] J. B. HARBORNE (Ed.) *Biochemistry of Phenolic Compounds*, Academic Press, New York (1964).
[15] P. MADHUSUDANAN NAIR and C. S. VAIDYANATHAN, *Phytochem.* 3, 235, 513 (1964).
[16] T. NAGASAWA, H. TAKAGI, K. KAWAKAMI, T. SUZUKI and Y. SAHASHI, *Agr. Biol. Chem. (Tokyo)* 25, 441 (1961).
[17] R. S. ANDREWS and J. B. PRIDHAM, *Nature* 205, 1213 (1965).

mainly derived from the leucoanthocyanins[18] and other flavonoids present in the testa. The possibility of some biogenetic relationship between these flavonoids and DOPA should perhaps be borne in mind.

MATERIALS AND METHODS

Plant materials. These were collected from the gardens of the University of London Botanical Supply Unit.

Preparation of pigments. Dried, blackened pods were soaked in distilled water containing a little detergent for 3 days in order to soften the tissues and remove extraneous surface materials. The pigment-containing outer layers were then carefully scraped off with a knife blade and extracted with cold acetone. The resulting grey powders contained white fibrous tissues which were, as far as possible, all removed with a forceps. Continuous successive Soxhlet extractions with methanol, pyridine, methanol, acetone and ether were then carried out and the powders air dried. Degradation of remaining polysaccharide, protein, etc. was effected by refluxing with 6 N HCl for 5 days and the black suspension was then filtered off and washed successively with water, methanol, ethanol, acetone and ether and again air dried. In the case of the *A. cicer* pods and *V. faba* flowers the preliminary soakings were omitted because these tissues were more succulent and readily extractable.

In order to synthesize melanins *in vitro*, potato phenolase was prepared by adding an equal volume of acetone (−20°) to fresh potato juice at 0°. The precipitate was centrifuged off and 0·1 M sodium phosphate buffer (pH 6·8) added. Insoluble material was spun down and substrates (0·02% L-tyrosine, DL-DOPA and dopamine) added to aliquots of the supernatant. Air was passed through the reaction mixtures at room temperature for 20 hr and the insoluble melanins isolated by centrifugation, washed with solvents and degraded with HCl as described above.

General methods. Infra-red measurements were made with an Infracord 137 using KBr discs.

Alkaline degradation of the pigments was carried out according to the method of Piattelli *et al.*[2] Separation of the products into phenols and phenolic carboxylic acids using bicarbonate was not attempted.

Phenolic compounds were examined on paper chromatograms using the following solvent systems: butanol-1: ethanol: water (40:11:19); butanol-1: acetic acid: water (60:15:25) and 2% aqueous HCl. Paper electrophoretic examinations were also made using 0·2 M sodium acetate (pH 5·2) and 8 mM sodium molybdate (pH 5·2) solutions as electrolytes.[19] Compounds were located on the papers with u.v. light, diazotized *p*-nitroaniline/NaOH and $AgNO_3$/NaOH spray reagents. The specimen of 5,6-dihydroxyindole which was used as a chromatographic standard was prepared from the di-*O*-benzyl derivative of 5,6-dihydroxyindole-2-carboxylic acid (a gift from Professor R. A. Nicolaus) by heating, to remove the carboxyl group, followed by hydrogenolysis to remove the benzyl groups.

Acknowledgements—We thank the Agricultural Research Council and United States Department of Agriculture for financial assistance and Dr. D. Steele for helpful discussions.

[18] M. J. ANTHISTLE, D. F. ASHDOWN and D. DICKINSON, *J. Sci. Food Agr.* **10**, 412 (1959).
[19] J. B. PRIDHAM, *J. Chromatog.* **2**, 605 (1959).

EDITED BOOKS*

C1 - C4

* One set only submitted.