1951. The financial assistance of the Agricultural Research Council of Great Britain during this period is gratefully acknowledged.

REFERENCES

- Appelmans, F., Wattiaux, R. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
- Appleyard, J. (1948). *Biochem. J.* **42**, 596.
- Axelrod, B. (1948a). *J. biol. Chem.* **176**, 296.
- Axelrod, B. (1948b). *J. biol. Chem.* **172**, 1.
- Borouhgs, H. (1954). *Arch. Biochem. Biophys.* **49**, 30.
- Bradfield, J. R. G. (1949). *Exp. Cell Res. Suppl.* **1**, 338.
- Brawerman, G. & Chargaff, E. (1954a). *Biochim. biophys. Acta*, **15**, 549.
- Brawerman, G. & Chargaff, E. (1954b). *Biochim. biophys. Acta*, **16**, 524.
- Delory, G. E., Wiberg, G. S. & Hetherington, M. (1955). *Canad. J. Biochem. Physiol.* **33**, 539.
- Dixon, M. (1949). *Multi-Enzyme Systems*. Cambridge University Press.
- Dixon, M. & Webb, E. C. (1953). *Brit. med. Bull.* **9**, 110.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hodge, A. J., Branster, M., Martin, E. M., Morton, R. K., McLean, J. D. & Mercer, F. V. (1956). *J. biophys. biochem. Cytol.* **2**, Suppl. 221.
- Hodge, A. J. & Morton, R. K. (1956). *Aust. J. Sci.* **19**, 73.
- London, M. & Hudson, P. B. (1955). *Biochem. biophys. Acta*, **17**, 485.
- Lundquist, F. (1947). *Acta physiol. scand.* **14**, 263.
- Møller, K. M. (1955). *Biochim. biophys. Acta*, **16**, 162.
- Moog, F. (1946). *Biol. Rev.* **21**, 41.
- Morton, R. K. (1952a). Ph.D. Thesis: University of Cambridge.
- Morton, R. K. (1952b). In *Résumés des Communications, II^e Congr. Int. Biochim., Paris*, p. 262.
- Morton, R. K. (1953). *Nature, Lond.*, **172**, 65.
- Morton, R. K. (1954a). *Biochem. J.* **57**, 231.
- Morton, R. K. (1954b). *Biochem. J.* **57**, 595.
- Morton, R. K. (1955a). *Biochem. J.* **61**, 232.
- Morton, R. K. (1955b). *Disc. Faraday Soc.* **20**, 149.
- Morton, R. K. (1958a). *Biochem. J.* **70**, 139.
- Morton, R. K. (1958b). *Biochem. J.* **70**, 134.
- Müller, Th., Rathlev, T. & Rosenberg, Th. (1956). *Biochim. biophys. Acta*, **19**, 563.
- Roche, J. (1950). In *The Enzymes*, vol. 1, part I, p. 473. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.
- Seegmiller, J. E. & Horecker, B. L. (1951). *J. biol. Chem.* **192**, 175.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157.
- Tsuboi, K. K. & Hudson, P. B. (1953). *Arch. Biochem. Biophys.* **43**, 339.
- Tunis, M. & Chargaff, E. (1956). *Biochim. biophys. Acta*, **21**, 204.

Cell-wall Polysaccharides of Myrobalans

BY D. E. HATHWAY AND J. W. T. SEAKINS

The British Leather Manufacturers Research Association, Milton Park, Egham, Surrey

(Received 20 December 1957)

Myrobalans are the dried fruit of an Indian tree, *Terminalia chebula* Retz.; they contain 43–53 % of tannin.

The present study was undertaken as there was no information relating to the cell-wall components, particularly as this is the first combretaceous plant to be investigated in this way. However, since the present work was carried out with dried-plant material, changes may have occurred in the polysaccharides during drying (cf. the rapid depletion of hemicelluloses in ripening banana; Barnell, 1943). This limitation applies with less emphasis to such polysaccharides as cellulose and polygalacturonic acid, at least some proportion of which would be expected to survive the changes occurring during the rapid drying process.

MATERIALS AND METHODS

Paper chromatography was effected in an all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated, and all solutions were evaporated in N_2 under reduced pressure at <35°.

Whole myrobalans, the dried fruit of *Terminalia chebula* Retz, grown in the Jubbulpore district of the Madhya Pradesh, were split open, the kernels were removed and the remaining tissue was pulverized (Hathway, 1956).

Preparation of cell-wall material

Pulverized myrobalans (100 g.) were extracted with several changes of fresh methanol in a Soxhlet apparatus until successive extracts were colourless. The residual material was then air-dried and ground to 60-mesh in a Wiley mill. The ground meal was then extracted with 89 % ethanol in a Soxhlet apparatus until sugars could not be detected in the last extract when treated with the anthrone reagent (Hathway, 1956). The final product was dried successively in a current of air, and over P_2O_5 at 20° in *vacuo* for 4 days. Yield, 18–19 g.

Cell-wall material prepared in this way contained polysaccharides (chiefly pectic material) originally present in the cell sap of the ripe fruit.

Fractionation of cell-wall material

The results of the analysis to which duplicate samples of cell-wall material were submitted are presented in Table 1. Water-soluble polysaccharides, equivalent to 'pectin', were removed with hot water, and, after chlorite delignification, the remaining 'holocellulose' was fractionated by

successive extraction with *n*- and 4*N*-KOH. The fractions of mixed polysaccharides obtained were all analysed for ash and, after hydrolysis, for their constituent monosaccharides and *D*-galacturonic acid.

A preliminary extraction of 10 g. of the cell-wall preparation with four successive volumes of 200 ml. of cold water removed most of the residual yellow pigment (600 mg.) and some pectin (270 mg.). The former appears as an un-numbered spot on the chromatogram shown as fig. 1 by Hathway (1956), where it has an R_f value of 0.22 in the first-way solvent system and remains adjacent to the ordinate in the second-way solvent system. The pectin was recovered free from this pigment by precipitation of the aqueous extract with 2.5 l. of ethanol.

The fraction which was insoluble in cold water was extracted with 750 ml. of water at 100° for 12 hr. The mixture was filtered through a sintered-glass funnel and the residue was washed successively with five 200 ml. portions of boiling water and a large volume of acetone. The yield of insoluble material was 6.58 g. The combined filtrate and washings were evaporated to 50 ml. and the polysaccharides were precipitated with ethanol. The yield of 'pectin' soluble in hot water was 2.64 g. 'Pectin' from the cold- and hot-water extracts was combined for the purpose of hydrolysis.

Extraction of the cell-wall material with aqueous solutions of ammonium oxalate or sodium polyphosphate (Allbright and Wilson Ltd., Oldbury, Wores.) did not remove a greater quantity of polysaccharide.

The fractionation of the water-insoluble material, and the hydrolysis of all the cell-wall fractions, were carried out by the methods of Jermyn & Isherwood (1956), and the hydrolysates were freeze-dried.

Analysis

Sugars in the hydrolysates. These were resolved by single-way paper chromatography effected by the descending method. Longitudinal strips were cut from each paper and sprayed with a *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) or *p*-anisidine phosphate solution in water-saturated butan-1-ol in order to locate the sugars on the unsprayed papers, which were cut into transverse strips for the purpose of elution and estimation (Flood, Hirst & Jones, 1947, 1948; Hawthorne, 1947; Jermyn & Isherwood, 1949). In the present work, the following solvent systems were employed: propan-1-ol-ethyl acetate-water (7:1:2, by vol.) (Albon & Gross, 1952; Hathway, 1956); ethyl acetate-acetic acid-water (3:1:3, by vol.) (Jermyn & Isherwood, 1949); the organic phase of benzene-butan-1-ol-pyridine-water (1:5:3:3, by vol.). The third solvent system, which resolves galactose and glucose, was used in the exploratory stages of the work to confirm the absence of galactose from the hydrolysates. Individual monosaccharides and *D*-galacturonic acid were estimated by a modified ferricyanide (Hagedorn & Jensen, 1923) method, unpublished details of which were kindly made available to us through the courtesy of Dr A. E. Flood.

The polygalacturonic acid content of the combined 'pectin' fractions was also determined by the calcium pectate method (Nanji & Norman, 1928).

Compounds other than polysaccharides in the cell wall

These were determined in each fraction, and the figure for the carbohydrate content was amended accordingly.

Some error is involved. Thus the possible presence of water-insoluble protein which is destroyed by chlorite oxidation (Wise, Murphy & D'Addieco, 1946) will enhance the apparent lignin figure, and a lower value was obtained by the hydrolysis method (Ritter, Seborg & Mitchell, 1932). The yellow pigment was estimated directly, and the possible presence of undetected impurities would lower this figure.

RESULTS

The cell-wall polysaccharides were fractionated by a series of analytical operations (Table 1) (Jermyn & Isherwood, 1956; O'Dwyer, 1923, 1926, 1928; Ritter & Kurth, 1933), which afforded fractions of mixed polysaccharides. These fractions were hydrolysed by the methods of Jermyn & Isherwood (1956) and the corresponding monosaccharides were separated by paper chromatography and estimated by established methods. For myrobalans it was readily established that the galacturonic acid can be accounted for as polygalacturonic acid, and the bulk of the glucose as cellulose. The remainder of the glucose is derived from starch, which is treated by the analytical procedure as a cell-wall constituent and which is consequently omitted from Table 2 which lists the cell-wall polysaccharides. The only other sugars present in the hydrolysates are traces of mannose and xylose, which correspond to the presence of small quantities of mannan and xylan in the cell wall. The polygalacturonic acid figure was checked by the calcium pectate method, and also by the isolation of the polyuronide. This procedure checked in the present context the overall assumption of Jermyn & Isherwood's (1956) methods. Hydrolysis of the isolated cellulose fraction showed that it was virtually pure polysaccharide. Cellulose-break-down products occur in the potassium hydroxide-soluble fractions, and these figures together with that for cellulose appear as 'holocellulose' in Table 2.

DISCUSSION

The present investigation of myrobalans deals with a large cell-wall fraction compared with that of such fruit as the apple (Smith, 1935; Norman, 1937; Kidd & West, 1939; Kidd, West, Griffiths & Potter, 1940; Echevin, 1941), pear (Hansen, 1939; Jermyn & Isherwood, 1956) and tomato (Clendenning, 1941). Moreover, the high cellulose and lignin contents of the cell wall, and the enormous cellular content of tannin extractives, are consistent with the fact that the dried fruit cannot be cut with a knife. Myrobalans which are picked before or at the climacteric (Chopra, 1939; Edwards, Badhwar & Dey, 1952; Howes, 1953) have subsequently undergone autolysis, accelerated by storage under tropical conditions, and this is confirmed by the presence of 'pectins' soluble in cold and hot water.

Table 1. Fractionation of cell-wall material from myrobalans

Figures are calculated as percentage of initial weight of moisture-free fruit. Sugars are expressed in the anhydro form.

* 2.2 by the method with sulphuric acid (see text).

Table 2. Total amount of each polysaccharide in the myrobalans cell-wall (minus the soluble glucosan)

Polysaccharide	Amount of each polysaccharide in the moisture-free fruit (%)
Holocellulose glucosan* (cellulose)	7.85
Mannan	0.05
Polygalacturonic acid†	1.9
Xylan	0.05
Total	9.85

* Estimation of starch in the n-KOH-soluble fraction showed that the glucosan of this fraction was distributed between starch and holocellulose in the ratio of 1:9.

† 1.5 by the calcium pectate method (see text).

It was first noticed by Frémy (1840) that insoluble protopectin which had been laid down in the primary cell walls during the growth phase of fruit was converted by protopectinase into soluble pectin during senescence. Later, Barnell (1943) found a depletion in the hemicelluloses of ripening banana, and in their outstanding study Jermyn & Isherwood (1956) reported that the principal labile materials during the breakdown of the pear cell wall are araban and galactan, together with a significant amount of holocellulose glucosan. The absence of arabinose and the presence of only a very small proportion of xylose in the hydrolysates of the cell-wall fractions show that hemicellulose components are virtually absent from dried myrobalans. This also applies to galactan. Although such hemicelluloses and galactan may have undergone rapid physiological change in the isolated

tissue during drying, the absence of galactose and the presence of only trace quantities of arabinose and xylose in the polyol fraction of the extractives (Hathway, 1956) suggests that the hemicellulose content of the cell wall may have never been high. In agreement with the fact that a significant quantity of holocellulose glucosan becomes labile during senescence, a proportion of myrobalans holocellulose glucosan was found in the soluble fractions.

It is difficult away from the country of origin to attempt to follow the changes which occur in the cell-wall polysaccharides during the ripening of myrobalans. The analytical methods used in the present work are suitable for this project, which is particularly worthwhile since this crop or 'hirda' is one of the most highly esteemed of the world's tanning materials.

SUMMARY

1. The cell-wall fraction of myrobalans is large and holocellulose glucosan accounts for 8% of the dry weight of the fruit and polygalacturonic acid for a further 2%. Galactans and hemicelluloses are virtually absent.

2. The changes which occur after abscission in senescent myrobalans give rise to soluble pectin and cellulose-breakdown products which may be separated from the cell wall of the dried fruit.

The authors thank the Director and Council of the British Leather Manufacturers' Research Association for their permission to publish this work. We are also indebted

to Dr A. E. Flood, of East Malling Research Station, who made available to us details of an improved ferricyanide method for the estimation of sugars.

REFERENCES

- Albon, N. & Gross, D. (1952). *Analyst*, **77**, 410.
 Barnell, H. S. (1943). *Ann. Bot., N.S.*, **7**, 297.
 Chopra, R. S. (1939). *Indian For.* **65**, 126.
 Clendenning, K. A. (1941). *Canad. J. Res.* **19C**, 500.
 Echevin, R. (1941). *C.R. Acad. Sci., Paris*, **213**, 458.
 Edwards, M. V. Badhwar, R. L. & Dey, A. C. (1952). *Indian For. Rec.* **1**, no. 2.
 Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). *Nature, Lond.*, **160**, 86.
 Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1948). *J. chem. Soc.* p. 1679.
 Frémy, E. (1840). *J. Pharm.* **26**, 368.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Hansen, E. (1939). *Plant Physiol.* **14**, 145.
 Hathway, D. E. (1956). *Biochem. J.* **63**, 380.
 Hawthorne, J. R. (1947). *Nature, Lond.*, **160**, 714.
 Hough, L., Jones, J. K. N. & Wadman, H. (1950). *J. chem. Soc.* p. 1702.
 Howes, F. N. (1953). In *Vegetable Tanning Materials*, p. 165. London: Butterworths Scientific Publications.
 Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
 Jermyn, M. A. & Isherwood, F. A. (1956). *Biochem. J.* **64**, 123.
 Kidd, F. & West, C. (1939). *New Phytol.* **38**, 105.
 Kidd, F., West, C., Griffiths, D. G. & Potter, N. A. (1940). *Ann. Bot., N.S.*, **4**, 1.
 Nanji, D. R. & Norman, A. G. (1928). *Biochem. J.* **22**, 596.
 Norman, A. G. (1937). In *Biochemistry of Cellulose*. Oxford: The Clarendon Press.
 O'Dwyer, M. H. (1923). *Biochem. J.* **17**, 501.
 O'Dwyer, M. H. (1926). *Biochem. J.* **20**, 656.
 O'Dwyer, M. H. (1928). *Biochem. J.* **22**, 381.
 Ritter, G. & Kurth, E. (1933). *Industr. Engng Chem. (Industr.)*, **25**, 1250.
 Ritter, G. J., Seborg, R. M. & Mitchell, R. L. (1932). *Industr. Engng Chem. (Anal.)*, **4**, 202.
 Smith, W. W. (1935). *Plant Physiol.* **10**, 587.
 Wise, L. E., Murphy, M. & D'Addieco, A. A. (1946). *Paper Tr. J.* **122**, 43.

The Influence of Tannins on the Degradation of Pectin by Pectinase Enzymes

BY D. E. HATHWAY AND J. W. T. SEAKINS

The British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey

(Received 20 December 1957)

When a high proportion of the cell-wall polysaccharides of myrobalans was found to consist of polygalacturonic acid (Hathway & Seakins, 1958), it became important to establish whether the available pectinases effect the degradation of pectin and low-methoxy pectin in the presence of myrobalans gallo- and ellagi-tannins. The effect of gambir-tannin extractives which contain (+)-catechin and phlobatannin on the enzymic degradation of pectin and low-methoxy pectin was also investigated.

MATERIALS AND METHODS

Paper chromatography was effected in all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated.

Pectin (apple) 240-grade (British Drug Houses Ltd.) was purified by repeated extraction with aq. 70% (v/v) ethanol, and the residue was dried at 35° and contained 18.6% of OMe.

Low-methoxy pectin was prepared by treatment of 50 g. of the commercial pectin with a mixture of 188 ml. of aq. 95% (v/v) ethanol and 62 ml. of N-NaOH for 20 min., and the precipitate obtained was washed with a mixture of 100 ml. of 95% ethanol, 10 ml. of water and 5 ml. of

N-HCl, and dried at 35°. Low-methoxy pectin contained <0.5% of OMe.

Pectin from the myrobalans cell-wall contained 16.0% of OMe.

Commercial pectinase preparations used in this work include Pectozyme (Norman Evans and Rais Ltd., Stockport, Ches.), Pectinol-100D (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), Pektinol klarlöslich doppelkonzentriert (Rohm und Haas A.-G., Darmstadt, Hessen, Germany). Since it was proposed to investigate the pectolytic action of pectic enzymes of fungal origin in the presence of hydrolysable tannins, it was necessary to determine whether they exhibit tannase activity, as tannase can be obtained from cultures of such fungi as *Aspergillus niger* which have been grown in the presence of myrobalans-tannin extractives (Freudenberg, 1921; Freudenberg & Vollbrecht, 1921). A 5% (w/v) soln. of myrobalans-tannin extractives was accordingly digested with a large excess of the three enzyme preparations at 25° for 1 week, when two-dimensional paper chromatograms of myrobalans extractives before and after enzyme treatment were compared (Hathway, 1956). The presence of such hydrolysable tannins as chebulagic and chebulinic acids and corilagin in approximately the same proportions on both sets of chromatograms suggests that these enzyme preparations are devoid of tannase activity. Known substrates of tannase include chebulinic acid and gallotannin.