72. *Carbohydrates of the Seeds of the Rubber Tree,* Hevea brasiliensis.

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The carbohydrate materials present in the endosperm, cotyledons, and shell of the seeds of the rubber tree, *Hevea brasiliensis*, have been examined by means of a graded extraction procedure, and the results of chromatographic and other analyses of the fractions so obtained are reported. The endosperm, cotyledons, and shells all contain a mixture of polysaccharides which gave on hydrolysis galactose, glucose, arabinose, xylose, rhamnose, and uronic acid units. (The percentages of these residues were 4, 60, 20, 8, 2, and 6, respectively for the endosperm; 5, 56, 22, 7, 3, and 7, respectively for the cotyledons; and 4, 11, 1, 80, trace, and 4, respectively for the shells.) The cold-water extract of the endosperm has been studied in detail and an araban-rich fraction isolated.

Recently, the starch present in the endosperm of the seeds of the rubber tree, *Hevea brasiliensis*, has been isolated, purified, and characterised.[1] Apart from this work and early studies on the glycoside, linamarin,[2] no detailed investigations of the carbohydrate content of the seeds of the rubber tree have been described.

In this paper, we report an investigation of the carbohydrate content of each of the principal parts of the seeds. The polysaccharides present in the endosperm, cotyledons, and shells have each been separated by successive extractions with cold water, hot water, and hot and cold 5% aqueous sodium hydroxide. The fractions obtained were analysed, and the results are shown in Tables 1—3. In addition, the cold-water extract of the endosperm has been examined in detail, and an arabinose-rich fraction isolated from it.

The seeds of the rubber tree contain considerable quantities of oil (50—60%), and graded extraction of the polysaccharide could not be attempted until this had been exhaustively extracted. (The presence of the glycoside, linamarin, was indicated in the oil-extractive from the endosperm.)

The heterogeneity of the polysaccharide material in each part of the seed was shown by the fact that successive extractions gave fractions which differed greatly in composition. It was found that both the endosperm and the cotyledons of the seed possessed a high protein-content; as only a proportion of this was water-soluble, contamination of the polysaccharide mixture at all stages of extraction was unavoidable.

The material from endosperm gave on hydrolysis galactose, glucose, arabinose, xylose, and uronic acid residues together with traces of rhamnose and fructose (see Table 1), and possessed a high percentage of starch material (fraction E1). The latter contains a very

[1] Greenwood and Robertson, *J.*, 1954, 3769.
[2] Gorter, *Rec. Trav. chim.*, 1912, **31**, 264; Dunstan and Henry, *Proc. Roy. Soc.*, 1904, *B*, **72**, 285; Fischer and Anger, *Ber.*, 1919, **52**, 854.

high proportion (48%) of protein and is extremely difficult to purify by methods unlikely to cause degradation of the starch components.[1]

The unhydrolysed cold-water extract (E2) when examined chromatographically was found to contain traces of glucose, maltose, sucrose, and polyfructoses, together comprising about 3% of the fraction. (No free sugars were present in any of the other fractions from the endosperm.) On hydrolysis, fraction E2 gave glucose, galactose, xylose, and arabinose. From this fraction, a polysaccharide was isolated which gave mainly arabinose on hydrolysis, indicating the presence of an araban. The fraction also contained a very high percentage of ash (see Experimental section). The large amount of glucose in the hot-water extract (E3) probably arises from traces of starch left after the aqueous leaching; the starch is extremely difficult to remove quantitatively.[1] In the case of this and subsequent fractions, extractions were continued until the yield of polysaccharide was negligible. Rhamnose appears in all fractions except (E2). The presence of this sugar is not unusual.[3,4]

TABLE 1. *Analyses of fractions isolated from rubber-seed endosperm (% of dry weight).*

Fraction	Yield	Ash *	Protein †	Uronic acid anhydride	Non-acidic polysaccharide ‡	Sugars obtained on hydrolysis					
						Gal	G	A	X	R	F
Benzene–methanol extractives	53	—	—	—	—	—	—	—	—	—	—
E1 (cold-water sediment)	23	5·3	48·4	2·0	44·3	0·9	40·4	2·6	0·4	0	0
E2 (,, extract)	3·5	27·3	21·4	4·4	46·9	3·7	25·0	15·0	1·9	0·9	0·4
E3 (hot-water ,,)	4·5	3·0	19·8	8·4	68·8	4·1	49·5	12·4	2·1	0·7	0
E4 (cold NaOH ,,)	11·3	15·5	27·5	2·7	54·3	5·9	23·3	13·6	9·3	2·2	0
E5 (hot ,, ,,)	1·4	12·4	17·0	0·6	70·0	1·4	31·5	26·0	9·0	2·1	0
E6 (residue) [a]	0·8	11·7	1·1	0	87·2	0·8	20·2	2·4	2·8	0	0

* Not sulphated. † % N × 6·25. ‡ Calc. by difference (for details see ref. 4).
Gal = galactose; G = glucose; A = arabinose; X = xylose; R = rhamnose; F = fructose.
[a] This fraction was only 26·2% hydrolysed under the conditions used (see text).

TABLE 2. *Analyses of fractions isolated from rubber-seed cotyledons (% of dry weight).*

Fraction	Yield	Ash *	Protein *	Uronic acid anhydride	Non-acidic polysaccharide *	Sugars obtained on hydrolysis *					
						Gal	G	A	X	R	F
Benzene–methanol extractives	65	—	—	—	—	—	—	—	—	—	—
C1 (cold-water sediment)	18·6	0·8	48·1	2·5	48·6	0·5	45·7	1·9	0·5	0	0
C2 (,, extract)	5·8	26·1	18·1	3·9	51·9	3·6	19·7	21·8	3·1	2·6	1·1
C3 (hot-water ,,)	2·6	7·0	32·7	18·1	42·2	3·4	19·0	16·8	1·3	1·7	0
C4 (cold NaOH ,,)	2·4	11·1	37·0	10·8	41·1	2·9	23·0	7·4	6·6	1·2	0
C5 (hot ,, ,,)	0·3	16·1	32·2	4·1	47·6	3·8	26·2	12·4	2·4	2·8	0
C6 (residue) [a]	1·2	2·4	1·6	2·4	93·6	0·7	13·3	2·8	1·5	0·3	0

* See footnotes to Table 1.
[a] This fraction was only 18·6% hydrolysed under the conditions used (see text).

In the alkaline extracts, glucose (probably from γ-celluloses) is predominant, but relatively large amounts of arabinose are present, and this sugar is present in greater amounts than xylose. Fraction (E6) was only 26·2% hydrolysed under the conditions described, but the residue on treatment with 72% sulphuric acid gave 95% of glucose, and was therefore cellulosic material.

Table 2 shows that the fractions from the cotyledons yield on hydrolysis the same sugars in approximately the same proportions. The unhydrolysed cold-water extract was found, when examined chromatographically, to contain no sucrose and no glucose, but polyfructoses and di- and tri-hexoses. Again the largest fraction was impure starch (C1). This contained the same large amount of protein (48%) and proved difficult to purify.

[3] Hirst, *J.*, 1949, 522.
[4] Anderson and Greenwood, *J.*, 1956, 220.

Investigations on this starch are being continued. The amount of arabinose predominates over that of xylose in all fractions. Fraction (C6) gave only glucose on hydrolysis with 72% sulphuric acid, and was probably cellulosic.

The same sugars are present in the fractions obtained from the rubber-seed shell, but their proportions differ greatly from those in the endosperm and cotyledons (see Table 3). Large amounts of lignin are present in all the fractions. From the analytical results, the cold-water sediment (S1) appears to consist merely of extremely fine powder from the shells (it gave no colour with iodine). Glucose does not appear in large amounts in the different fractions (compare Tables 1 and 2). Araban is relatively non-existent, but large amounts of both water- and alkali-soluble xylan exist in the shells: they form the main portion of each fraction. Fraction (S6) was again shown to be mainly cellulosic material.

TABLE 3. *Analyses of fractions isolated from rubber-seed shells (% of dry weight).*

Fraction	Yield	Ash *	Protein *	Uronic acid anhydride	Lignin	Non-acidic polysaccharide *	Sugars obtained on hydrolysis *				
							Gal	G	A	X	R
Benzene–methanol extractives	18·8	—	—	—	—	—	—	—	—	—	—
S1 (cold-water sediment)	1·0	10·4	4·1	3·2	14·8	67·5	2·7	29·7	0	35·1	0
S2 (,, extract)	0·5	17·6	7·1	4·6	8·6	62·1	1·8	5·6	0	54·7	0
S3 (hot-water ,,)	1·5	10·0	4·8	4·9	26·7	53·6	2·2	3·8	2·6	45·0	0
S4a (cold NaOH ,,)	2·4	5·1	0·7	7·8	52·3	34·1	2·7	3·4	0	28·0	0
S4b (,, ,, ppt.)	10·4	10·3	3·8	2·7	65·5	17·7	0	0·5	0·7	16·3	0·2
S5a (hot NaOH extract)	5·2	14·8	4·8	4·5	34·3	41·6	2·9	2·2	0·4	35·3	0·8
S5b (,, ,, ppt.)	8·7	0·7	1·1	3·3	7·9	87·0	1·7	3·5	0	81·8	0
S6 (residue) [a]	41·0	0·2	0·5	0·7	44·2	54·4	0·4	2·5	0	11·1	0

* See footnotes to Table 1.

[a] This fraction was only 14% hydrolysed under the conditions used (see text).

The cold-water extract of the endosperm (E2) was then studied in more detail, and attempts made to fractionate the polysaccharide mixture. Amino-sugars were shown to be absent and therefore the nitrogen content most probably arises from protein. The large amount of this caused difficulties. Attempts to precipitate it as a complex and to denature it in solution were unsuccessful; large amounts of polysaccharide were always absorbed on the protein complex. Although treatment of the solution with resin caused a reduction in the amount of protein, it involved the simultaneous loss of one-third of the polysaccharide material. When attempts were made to remove protein by acetylation, some was still present in the acetylated product. The latter, however, on chromatographic examination was found to be an arabinose-rich product, and indicated the presence of an araban. The uronic acid content of the fraction was shown to be due to the presence of a polyuronide, giving rise to aldobiuronic acids on hydrolysis, and not to pectic acid. Chromatographic examination of the hydrolysed barium aldobiuronate indicated that glucuronic acid was present. After several methods of fractionation had been attempted, it was shown that, when the main bulk of the fraction had been precipitated from aqueous solution by ethanol, the material remaining in the supernatant liquors yielded mainly arabinose on hydrolysis with traces of galactose and xylose, uronic acids being absent.

EXPERIMENTAL

Before analyses, samples were dried *in vacuo* for several hours at 80°. Solutions were concentrated under reduced pressure at 40°. Percentages of nitrogen were determined by replicate semimicro-Kjeldahl determinations. Estimations of uronic acid anhydride were made by McCready's method,[5] whilst determinations of " acid lignin " were made by Bamford

[5] McCready, Swenson, and Maclay, *Analyt. Chem.*, 1946, **18**, 290.

and Campbell's method [6] with the modification that the polysaccharide material was *shaken* with 72% sulphuric acid at room temperature for 6 hr. Separations of sugars were carried out by partition chromatography (Whatman No. 1 filter paper; descending method) with the following solvents: (a) *for free sugars*, butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3, v/v; top layer: development time, 48 hr. at 21·5°; (b) *for uronic acids*, butan-1-ol–formic acid–water (4 : 2 : 4, v/v): development time, 48 hr. at 21·5°. Aniline oxalate spray was used to detect aldoses, whilst urea oxalate was used for ketoses. Unless otherwise stated, polysaccharides were hydrolysed by 2% sulphuric acid in a sealed tube at 98° for 7 hr.

Preliminary Separation of Seed Materials.—After removal of the shells, the endosperm from each seed was split by hand and the cotyledons removed. The last two parts of the seed were then placed under ethanol to inhibit enzymic action.

Extraction of Oil.—(a) *From the endosperm.* This was effected as previously described [1] (Found on defatted material: ash, 6·2; protein, 44·3; uronic acid anhydride, 2·9%). On hydrolysis, the ratio of sugars found was galactose : glucose : arabinose : xylose : rhamnose = 5 : 64 : 21 : 8 : 2. Hence, by calculation, the percentage composition of the polysaccharide material present is: galactose, 4; glucose, 60; arabinose, 20; xylose, 8; rhamnose, 2; uronic acid anhydride, 6%.

(b) *From the cotyledons.* As in (a) (Found on defatted material: ash, 2·7; protein, 18·3; uronic acid anhydride, 5·6%). On hydrolysis, the ratio of sugars found was galactose : glucose : arabinose : xylose : rhamnose = 6 : 60 : 24 : 7 : 3. Hence the percentage composition of the polysaccharide material present is: galactose, 5; glucose, 56; arabinose, 22; xylose, 7; rhamnose, 3; uronic acid anhydride, 7%.

(c) *From the shells.* The shells were ground in the hammer mill to yield a fine powder, which was treated as above (Found on defatted material: ash, 0·5; protein, 1·75; uronic acid anhydride, 2·1; lignin, 45%). On hydrolysis with 90% formic acid,[7] the ratio of sugars found was galactose : glucose : arabinose : xylose = 4 : 12 : 1 : 83. Hence, the percentage composition of the polysaccharide material present is: galactose, 4; glucose, 11; arabinose, 1; xylose, 80; uronic acid anhydride, 4%.

Extraction Scheme.—The endosperm, cotyledons, and shells were subjected to the graded extraction scheme previously outlined [4, 8] using cold and hot water, followed by cold 5% and hot 5% alkali. For the endosperm this gave fractions E1—6 (94% recovery of defatted material); for the cotyledons, fractions C1—6 (88% recovery); and for the shells, fractions S1—6 (87% recovery). Only in the case of the shells did neutralisation of the alkaline extracts give a precipitate (*i.e.*, fractions S4b and S5b).

Analysis of the Fractions.—Qualitative and quantitative estimations of the sugars liberated on hydrolysis were carried out as previously described.[4, 8] The presence of rhamnose and uronic acids was confirmed as before.[4] For fractions S1—S6, the large amount of unhydrolysed material was rehydrolysed with 5% hydrochloric acid at 98° in a sealed tube. No further sugars were liberated by this treatment.

Examination of the Cold-water Extract from the Endosperm (E2).—(a) *Tests for amino-sugars.* Elson and Morgan's method [9] was used. All the reagents were carefully purified (ethanol by Winkler's method; [10] acetylacetone by Claisen's method; [11] *p*-dimethylaminobenzaldehyde by fractional precipitation from hydrochloric acid solution). It was found that the *unhydrolysed* protein-contaminated fraction gave a positive test (a cherry-red colour) identical with that obtained with glucosamine. The Elson–Morgan test is therefore not specific: this is in agreement with Vasseur and Immers's work.[12] The *hydrolysed* fraction did not give any reaction, and hence amino-sugars were absent.

(b) *Ash-content.* Ca^{++}, Fe^{+++} (trace), Mg^{++}, K^{+}, Na^{+}, PO_4^{3-} (very large amount), SO_4^{--}, and Cl^{-} (trace) were present. A quantitative estimation showed that 2·5% of the fraction was sulphate, which was further shown to be completely unattached to polysaccharide material.

(c) *Attempted removal of protein.* Heat-coagulation (15 min. at 90°) gave material containing 88% of protein, whilst the remaining liquors contained 15%. The formation of lead

[6] Bamford and Campbell, *Biochem. J.*, 1936, **30**, 419.
[7] Jones, *J.*, 1950, 3292.
[8] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.
[9] Elson and Morgan, *Biochem. J.*, 1933, **27**, 1824.
[10] Winkler, *Ber.*, 1905, **38**, 3612.
[11] Claisen, *Annalen*, 1893, **227**, 162.
[12] Vasseur and Immers, *Arkiv Kemi*, 1949, **1**, 253.

acetate–, trichloracetic acid–, and picric acid–protein complexes caused complete co-precipitation of polysaccharide, whilst the Sevag method [13] was equally unsuccessful. Passage of a 1% aqueous solution through a " Zeo-Karb 215 " resin decreased the protein content to 5·2%, but was accompanied by retention of one-third of the polysaccharide on the column. A final attempt was made to remove protein by acetylating a portion (15 g.) with pyridine (200 ml.) and acetic anhydride (150 ml.) for 4 days at room temperature. After removal of insoluble material on the centrifuge, the acetate was isolated and purified. The insoluble residue was reacetylated with formamide as a dispersive agent. The total yield of acetate was 1·4 g. (Insoluble portion, 11 g.) {Found : $[\alpha]_D^{17}$ $-92°$ (*c* 0·5 in $CHCl_3$); Ac, 41; protein, 5%; hydrolysis gave galactose : arabinose : xylose = 1 : 20 : 1}.

(d) *Investigation of the polyuronide.* Pectic material was probably absent, since (i) no precipitate was formed on the addition of a saturated solution of calcium chloride, and (ii) the material had no methoxyl content.

The aldobiuronic acid content of the fraction (0·5 g.) was investigated by hydrolysis at 100° with 3% aqueous oxalic acid (25 ml.) for 12 hr. After neutralisation with barium carbonate, the barium salt of the aldobiuronic acid was isolated by pouring the concentrated liquors (1 ml.) into alcohol (25 ml.). The salt (10 mg.) was then hydrolysed at 100° with 9% sulphuric acid (0·4 ml.) for 20 hr. The solution was diluted (5 ml.) and neutralised with barium carbonate, and half of the resultant solution concentrated and examined chromatographically : glucose and arabinose were detected. The other portion was de-ionised by shaking it with resin, concentrated to a syrup, and examined chromatographically with solvent (*c*). Glucuronic acid, glucurone, glucose, and arabinose were detected.

Fractionation Experiments on (*E*2).—(a) *Copper complex.* Chromatographic examination of the complex (formed by the addition of saturated copper acetate to the aqueous solution) showed that only arabinose and amino-acids were present on hydrolysis (Yield, 25% of fraction; uronic acid anhydride, 0%).

(b) *Glacial acetic acid.* Morris's method [13] was used. Chromatographic analysis showed that an arabinose-rich fraction was obtained from the 88% glacial acetic acid supernatant liquid.

(c) *Ethanol fractionation.* Ethanol was added to a 1% aqueous solution of the fraction to form precipitates at 67, 75, 80, and 83% of alcohol. Chromatographic examination of these and the material remaining in supernatant layers showed that the supernatant layer (32% yield) contained arabinose-rich material. The alcohol-fractionation method was repeated on a large-scale (9 g. in 300 ml. of water) to yield 2·35 g. of supernatant material (Found : protein, 27·5; uronic acid anhydride, 0%; galactose : arabinose : xylose = 1 : 20 : trace respectively).

Examination of the Linamarin.—A portion of the benzene–methanol extract from the endosperm was purified by Gorter's method [2] to yield a pale yellow syrup, which did not crystallise on standing. It contained no simple reducing sugars and on hydrolysis yielded 73% of glucose (Calc. for $C_9H_{17}O_6N$: 76·6%), acetone (iodoform test), and hydrogen cyanide (" Prussian Blue " test). It had $[\alpha]_D^{16}$ $-27°$ (*c* 1 in H_2O) (cf. $[\alpha]_D^{16}$ $-27·7°$; ref. 2). Methylation followed by chromatographic examination of the hydrolysis product showed the presence of 2 : 3 : 4 : 6-tetra-*O*-methylglucose only.

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[13] Morris, *J. Biol. Chem.*, 1942, **142**, 881.