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Hydrolysis of the Soluble Pentosans of Wheat Flour and *Rhodymenia palmata* by Ruminal Micro-organisms

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It has been known for some time that pentosans in the food are to a large extent decomposed during passage through the rumen of sheep (Marshall, 1949; Heald, 1953). This microbial activity enables the ruminant to make use of pentosans and other hemicelluloses which, like cellulose, are hardly, if at all, available to non-ruminants. Heald (1952) also isolated some xylose-fermenting strains of *Escherichia coli* from the rumen but, when the work about to be described was undertaken, nothing was known of the bacteria in the rumen responsible for fermenting the pentosans themselves. Two species of pentosan-fermenting bacteria have recently been found in the rumen; both appear to be hitherto undescribed species of nutritionally exacting, obligately anaerobic, Gram-negative, curved rods

(Bryant & Small, 1956; Doetsch, Howard, Mann & Oxford, 1957).

To assist in an understanding of the processes of pentosan digestion in the sheep rumen, experiments were undertaken *in vitro* with suspensions of the natural mixed culture of bacteria found in the rumen of a sheep which was maintained on a standard diet containing a fair amount of pentosan. It was considered that pentosans which were water-soluble and could be obtained fairly readily in a state of purity would present the most convenient type of substrate for preliminary studies. Two such materials have been examined so far, the soluble pentosan of wheat flour and the soluble xylan of the red seaweed *Rhodymenia palmata*. The former was first isolated by Perlin (1951a) from commercial

wheat flour. Perlin (1951b) showed the polysaccharide to have a main chain of D-xylopyranose units, linked β -1:4, with single L-arabofuranoside units attached along the chain at positions 2 and 3 of the xylose. About 300 anhydropentose units were present in each molecule. In the present work, material very similar in properties to Perlin's pentosan has been obtained by cold-water extraction of unbleached untreated wheat flour. In preparing large amounts of hexosan-free material for these studies a new method of purification has been developed, based on the observation that the hexosan present in the crude material is susceptible to the hydrolytic action of saliva whereas the pentosan component is not. This enzymic purification of the pentosan affords proof, additional to Perlin's (1951a) chemical separation, that the pentose and hexose in the water-soluble polysaccharides of wheat flour exist in separate pentosans and hexosans, and not in mixed polysaccharides.

The xylan of *Rhodymenia palmata* was first isolated by Barry & Dillon (1940), and subsequent chemical investigations have been reported by Percival & Chanda (1950), Barry, Dillon, Hawkins & O'Colla (1950) and Hirst (1955). The polysaccharide molecule is relatively small, consisting of only 16 or 17 xylopyranose units. The unique feature of this xylan is that although most of the xylose units are linked in β -1:4 fashion as in xylans generally, about one linkage in five is of the β -1:3 type. Nothing appears to be known about the distribution of these linkages along the chain.

The present communication is concerned with the hydrolytic activities of toluene-treated suspensions of mixed rumen bacteria, particular attention being paid to the oligosaccharide intermediates in the hydrolysis of the two substrates. Some hitherto unknown xylose oligosaccharides have been obtained from the *Rhodymenia* xylan, and their chemical constitutions have been investigated. It is proposed that the name *rhodymenan* be given to this unique polysaccharide, and that the characteristic disaccharide obtained from it, containing two xylose residues linked β -1:3, and which is described below, be known as *rhodymenabiose*. The contribution which this investigation makes to knowledge of the structure of rhodymenan is considered. The only xylose oligosaccharides known hitherto have all belonged to the β -1:4 series (Whistler & Tu, 1952), and the simple names xylobiose, xylotriose, etc., have already been appropriated for these substances. That part of the present work dealing with the wheat-flour pentosan has already been briefly reported (Howard, 1955).

METHODS AND MATERIALS

Sheep used as a source of rumen bacteria. The sheep used throughout this investigation was a Cheviot wether, no. 879, fitted with a rumen cannula, and fed daily with a mixture of

hay (750 g.) and concentrates (450 g., containing chiefly ground maize, crushed oats and bran). This was the sheep from which Doetsch *et al.* (1957) isolated the pentosan-fermenting organism *Bacteriodes amylogenes*.

Preparation of suspensions of rumen bacteria. Quantities of approximately 1 l. of rumen liquor were removed through the cannula about 2 hr. after feeding, and mixed with sodium sulphide solution to give a final concentration of 0.02% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in order to maintain anaerobic conditions during manipulation. Larger food particles were removed by straining through several layers of surgical gauze; Protozoa and small food particles were removed by centrifuging at 300 g for 1 min. The supernatant solution, containing large numbers of different eubacteria, but only very few of the smaller Protozoa and larger bacteria, was spun at 4500 g for 30 min. The almost clear supernatant was discarded, and the cells were resuspended in the buffer solution described by Elsdon (1946), ammonium salts being omitted (0.2M- NaHCO_3 , 100 ml.; 0.154M-KCl, 4 ml.; 0.11M- CaCl_2 , 3 ml.; 0.154M- KH_2PO_4 , 1 ml.; 0.154M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml.; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.02%. The solution was saturated with CO_2 after mixing, and then had pH 6.8).

Preparation of wheat-flour pentosan. Unbleached, untreated wheat flour (500 g.; supplied by Thomas Hulbert and Sons Ltd., New Era Flour Mills, Manchester) was shaken mechanically for 2 hr. with water (1 l.). The extract was separated by centrifuging, adjusted to pH 3 with HCl and filtered through glass wool to remove a little scum. Ethanol was added to the filtrate with stirring to a final concentration of 70% (v/v). The white precipitate was collected, and washed several times with ethanol, then with ether and dried *in vacuo*. The yield of crude pentosan was about 6 g. The ratio of pentosan:hexosan was approx. 1.5:1.

Crude pentosan (10 g.) was ground to a fine powder, mixed with water (300 ml.) and stored overnight in the refrigerator. Solution of still undissolved material was then completed by warming. To this was added human saliva (150 ml.) collected under toluene and centrifuged at 2500 g for 30 min. The mixture of pentosan and saliva was covered with toluene and incubated at 37° for 24 hr. with occasional shaking.

Trichloroacetic acid (10%, 400 ml.) was added to the mixture after incubation, and the flask stored at 0° overnight. The precipitate was removed by centrifuging and washed with a little cold 5% trichloroacetic acid solution. To the combined solution and washings (850 ml.) ethanol (2 l.) was added with stirring. The precipitate was washed with 70% (v/v) ethanol (3×150 ml.), then with absolute ethanol and finally with ether. The yield of pentosan was 4.35 g., i.e. 0.5% of the original flour [Found: N (Kjeldahl), 0.8%]. An acid hydrolysate, when tested chromatographically, was found to contain chiefly xylose and arabinose, in that order. Traces of glucose and galactose were also present. The hydrolysate contained 96% of reducing sugar, calculated as anhydropentose.

Preparation of rhodymenan. *Rhodymenia palmata* ('dulce'), as purchased in the Aberdeen market, was found to be almost free from other plants, and was used without sorting or cleaning. A quantity was obtained and stored at -20° until required. The thawed-out weed (1 kg.) was first passed through a kitchen mincer, and portions of the mince (100 g.) were mixed for 5 min. in a Waring Blendor with ice-cold 0.2N-HCl (300 ml.). The extract was separated by a short centrifuging and filtered through glass wool into mechanically stirred acetone (2 vol.).

A further quantity of xylan was obtained from the acid-extracted weed by suspending in water (2.5 l.), neutralizing with 2N-NaOH and heating to 70° for 2 hr. The hot extract was separated by filtration at the pump, and the xylan precipitated with acetone. The crude materials obtained from the two extractions were combined, washed with acetone and then dissolved in 0.2N-NaOH (4 l.) by shaking. A little insoluble matter was removed by centrifuging, and the clear solution was mixed with Fehling's solution (400 ml.) with vigorous stirring. The gelatinous lump of copper complex was removed, washed twice by thorough kneading in water and dissolved by shaking in 0.2N-HCl (3 l.). The solution was poured into acetone (6 l.) and the white precipitate washed with acetone, first by decantation and finally on a Büchner filter until the washings were free from the orange-coloured complex copper ion. The yield was 25 g., equivalent to 2.5% of the wet weed. The copper content was 0.002%, the anhydroxylose content 100%; other sugars were absent.

Incubation of substrates with the bacterial suspension. The materials were ground to a fine powder and dissolved in a portion of the buffer solution by warming. The solution was mixed with the necessary volume of the bacterial suspension, covered with a thin layer of toluene and flushed with CO₂ to remove air. The stoppered vessel was incubated at 38° with occasional shaking. The quantity of bacterial suspension used, and the time of incubation in the different experiments, are mentioned in the appropriate places below. Samples withdrawn for reducing sugar determination were immediately deproteinized and reducing values determined, by the methods of Somogyi (1945*b*, *a* respectively). Results were expressed in terms of xylose. The bacterial suspensions were always used within 2 hr. of taking the rumen liquor from the sheep.

Chromatographic methods. Solutions which had been clarified as described in the preceding paragraph were de-ionized by passage through columns of Amberlite IR-120 (H) and IR-4B (OH) ion-exchange resins, or, with small samples, were treated in the tubes with small amounts of the two resins together. De-ionized samples which were too dilute were concentrated in a vacuum desiccator over anhydrous CaCl₂. Samples were applied to sheets of Whatman no. 1 paper and developed by the descending technique. The three solvent mixtures employed for development were: (A) ethyl acetate-pyridine-water (10:4:3, by vol.); (B) butanol-pyridine-water (10:3:3, by vol.); (C) upper layer of butanol-acetic acid-water (4:1:5, by vol.). The spray reagent was prepared by mixing equal volumes of 0.1M-aniline and 0.1M-phosphoric acid, both in water-saturated butanol. In order to separate oligosaccharides it was necessary to allow the solvent to run off the paper, so that R_F values could not be determined. R_x , the ratio of the distance travelled by the substance to the distance travelled by xylose on the same paper, was found to be a convenient value to measure. In this paper the values of R_x , for a substance in the three solvent mixtures (A), (B) and (C), are shown in that order.

Paper electrophoresis of oligosaccharides. Electrophoresis was carried out in 0.2M-borate buffer, pH 10, on Whatman no. 3MM paper, in an apparatus similar to that of Kunkel & Tiselius (1951). The applied p.d. was 230 v, giving a current of 6-7 ma. The duration of electrophoresis was 5 or 18 hr. according to the mobility of the substances being examined. Substances were detected on the paper after drying, by the aniline phosphate spray. In calculating M_x (mobility with

respect to xylose), correction was made for electroendosmotic flow by including *N*-2:4-dinitrophenylethanolamine as a coloured, non-mobile marker (Mould & Synge, 1954).

Large-scale hydrolysis of wheat-flour pentosan by rumen bacteria. This was carried out according to the general procedure described above. The buffer solution (450 ml.) contained wheat-flour pentosan (9.4 g.) and the bacteria from 800 ml. of rumen liquor. After incubation for 6 hr., the reducing value of the mixture corresponded to about 45% hydrolysis. The reaction was arrested and the mixture clarified, by the addition of 5% ZnSO₄ solution (50 ml.) followed by hot saturated Ba(OH)₂ until no more precipitate formed. Centrifuging and filtration yielded a clear, almost colourless solution which was de-ionized and concentrated *in vacuo* to 50 ml. This was added to the top of a column (400 mm. × 37 mm.) made from a mixture of equal parts of charcoal (B.D.H., acid-washed for decolorizing purposes) and Celite 535 (Johns Manville Co., New York) (Whistler & Durso, 1950). Aqueous ethanol was used as eluent, the concentration of ethanol being increased stepwise as required. The eluate was collected in fractions of 100 ml. each. Each fraction was tested chromatographically and also for total pentose by a modification of Drury's (1948) method. Appropriate fractions of the eluate were combined, concentrated *in vacuo*, filtered through a grade 5 sintered-glass filter, and dried. A portion of each of the combined fractions was hydrolysed by N-H₂SO₄ for 1 hr. at 100° and the neutralized hydrolysates were examined chromatographically.

Large-scale hydrolysis of rhodymenan. The xylan (5 g.) and a bacterial suspension prepared from 500 ml. of rumen liquor were incubated together in buffer solution (250 ml.). The treatment of the hydrolysate was the same as described for the wheat-flour pentosan.

Determination of molecular weights of oligosaccharides. Substances to be tested were dried *in vacuo* at 100° and the molecular weights determined by the hypoidite method (Hirst, Hough & Jones, 1949).

Periodate oxidation of oligosaccharides. Quantities of approx. 2 mg. of the substances were oxidized in Warburg respirometers by the method of Perlin (1954) at a temperature of 18° and pH 5.7. The solutions remaining after titrating periodate were de-ionized and hydrolysed as described by Bishop (1956). The hydrolysates were tested for pentose both quantitatively (Drury, 1948) and qualitatively (paper chromatography in solvent mixture B).

Acid hydrolysis of carbohydrates. Complete hydrolysis was achieved by heating the substances with N-H₂SO₄ in sealed tubes in boiling water for 1 or 2 hr. as required. Partial hydrolysis was achieved by similar treatment with 0.1N-H₂SO₄ for 0.5 or 1 hr. Acid was neutralized with BaCO₃ before the hydrolysates were chromatographed.

EXPERIMENTAL AND RESULTS

Tests for oligosaccharide synthesis by the bacterial suspension. The bacterial suspension was incubated under the conditions described above in a solution containing 0.3% each of L-arabinose and D-xylose. Samples removed at intervals during 24 hr. and examined chromatographically were found to contain no sugars other than arabinose and xylose. In a similar experiment with xylobiose (see below) no sugars other than the starting material and xylose were detected after incubation.

Complete hydrolysis of the polysaccharides by the bacterial suspension. Solutions of the wheat-flour pentosan or rhodymenan (0.5%) were incubated with the bacterial suspension. After 18 hr. the reducing value in each case was the same as that of an equivalent amount of polysaccharide after complete acid hydrolysis (2 hr.). That hydrolysis by the bacterial suspension was almost complete was confirmed by chromatography; the wheat-flour pentosan hydrolysate contained arabinose, xylose and a barely detectable trace of

xylobiose; the rhodymenan hydrolysate contained, in addition to xylose, barely detectable traces of xylobiose and 3²- β -xylosylxylobiose (see below). For an explanation of this terminology see *Biochem. J.* (1957), 65, 8.

Course of hydrolysis of wheat-flour pentosan by the bacterial suspension. In order to follow the course of the hydrolysis in detail, wheat-flour pentosan (0.5%, w/v soln.) was incubated with the bacterial suspension and samples taken at frequent intervals were examined chromatographically. The first low-mol.wt. substance formed was arabinose, followed by the lower xylose oligosaccharides and finally by xylose itself (Fig. 1, Z). As hydrolysis proceeded the intensity of the colour reaction of the spots on the starting line increased to a maximum and then declined, concurrently with formation of appreciable amounts of xylose. Except in the final stages of the hydrolysis, arabinose was by far the most abundant product of low mol.wt. present. A chromatogram of rhodymenan after partial hydrolysis by rumen bacteria is shown in Fig. 1, Y. Comparison with Fig. 1, Z shows that the rhodymenan hydrolysate contains a number of substances not found in the wheat-flour pentosan hydrolysate. Thus although the spot (c) is hardly to be distinguished from arabinose it must represent another sugar, as arabinose is not a constituent of rhodymenan. Similarly, the spots (f) and (h) have no counterpart in the hydrolysate of the wheat-flour pentosan.

Fig. 1. Paper chromatogram of partially hydrolysed pentosans. X, Rhodymenan, 0.1N-H₂SO₄; Y, rhodymenan, rumen bacteria; Z, wheat-flour pentosan, rumen bacteria. Solvent mixture B. Key to spots: (a) xylose; (b) arabinose; (c) rhodymenabiose; (d) glucose (from bacterial cells by autolysis); (e) xylobiose; (f) 3²- β -xylosylxylobiose; (g) xylotriose; (h) xylose tetrasaccharide containing 1:3 linkage; (i) xylotetraose and higher oligosaccharides.

Oligosaccharides from large-scale hydrolysate of wheat-flour pentosan

Fractionation of the partial hydrolysate on a charcoal column yielded 12 fractions, as shown in Table 1.

Fraction 1. This was a syrup, which was shown by paper chromatography to contain a large proportion of arabinose, rather less xylose and a trace of glucose. On long standing it partly crystallized. The solid product was thoroughly drained on a porous tile and recrystallized from ethanol. It had m.p. 155°, not depressed on admixture with authentic L-arabinose, m.p. 158°.

Fraction 2. This was a syrup which did not crystallize until after seeding with crystals of authentic xylobiose. Recrystallized from aq. ethanol, the substance had m.p. 185°, $[\alpha]_D^{20} - 25^\circ$ in water [Whistler & Tu (1951) found m.p. 187°, $[\alpha]_D - 25.5^\circ$] (Found: mol.wt. 298, 299. Calc. for C₁₀H₁₈O₆: 282). R_F values: 0.83, 0.39, 0.33.

Fraction 3. This was a syrup which could not be crystallized, but was chromatographically homogeneous; R_F values: 0.55, 0.12, 0.09, chromatographically indistinguishable in all three solvent systems from authentic xylotriose, and having $[\alpha]_D^{20} - 45^\circ$ in water [Whistler & Tu (1951) found -47° and Bishop (1955) found -44.4°] (Found: mol.wt. 426. Calc. for C₁₅H₂₆O₁₁: 414).

Fraction 4. This was a syrup containing a little xylotriose and a major, less-mobile component (R_F values: 0.28, 0.03, 0.03), very probably xylotetraose.

Fractions 5 and 6. These contained, in addition to the triose and tetraose of the preceding fractions, small and moderate amounts respectively of a less-mobile constituent (R_F values: 0.15, 0.02, 0.01), very probably xylopentaose.

Oligosaccharides from large-scale hydrolysate of rhodymenan

Charcoal-column separation of the hydrolysate yielded eight fractions, as shown in Table 2.

Table 1. *Charcoal-column fractionation of wheat-flour pentosan (9.4 g.) after partial hydrolysis by rumen bacteria in the presence of toluene*

Key to symbols in column 5: + + + + +, more arabinose than xylose; + + + +, almost as much arabinose as xylose; + + +, moderate proportion of arabinose; + +, trace of arabinose; +, barely detectable trace of arabinose; 0, no arabinose.

Fraction no.	Concn. of ethanol (% v/v) in eluting agent	Vol. of eluate (l.)	Wt. of fraction (g.)	Arabinose content of hydrolysed fraction
1	0	2.3	4.39	+ + + + +
2	5	1.8	0.60	0
3	10	4.6	0.43	0
4	18	1.3	0.12	+
5	21	1.3	0.17	+
6	21	1.1	0.26	+
7	24	1.4	0.21	+ +
8	24	2.1	0.21	+ + +
9	27	1.1	0.14	+ + +
10	27	1.2	0.12	+ + +
11	30	3.8	0.32	+ + +
12	30	3.7	0.82	+ + + +
		Total	7.79 (83%)	

Table 2. *Charcoal-column fractionation of rhodymenan (5 g.) after partial hydrolysis by rumen bacteria in the presence of toluene*

Fraction no.	Concn. of ethanol (% v/v) in eluting agent	Vol. of eluate (l.)	Wt. of fraction (g.)
1	0	1.2	2.20
2	5	1.0	0.30
3	10	0.5	0.07
4	10	0.6	0.17
5	10, 15	2.1	0.31
6	18	2.2	0.31
7	24	2.0	0.09
8	30-50	4.0	0.33
		Total	3.78 (76%)

Fraction 1. This was a syrup which readily crystallized. Recrystallized from ethanol with charcoal treatment, it had $[\alpha]_D^{20} + 19^\circ$ in water (c, 3.5), m.p. 148° , not depressed on admixture with authentic D-xylose, m.p. 147° .

Fraction 2. This was a syrup, which did not crystallize until seeded with authentic xylobiose. It crystallized from aq. ethanol as needles, m.p. 190° , $[\alpha]_D - 25.8^\circ$ in water; R_x values: 0.82, 0.39, 0.35; M_n value: 0.16 (Found: mol.wt., 286, 299. Calc. for $C_{10}H_{18}O_6$: 282). Complete acid hydrolysis yielded only xylose, and chromatograms of partial acid hydrolysates showed no sugars other than the starting material and xylose.

Fraction 3. The small amount of syrup partially crystallized in needles on standing and was purified by extraction with ethanol. *Rhodymenabiose* had $[\alpha]_D^{20} - 18.4 \pm 0.6^\circ$ in water (c, 3.25); R_x values: 1.05, 0.77, 0.69; M_n value: 0.60 (Found: C, 42.0; H, 6.7; mol.wt. 300. $C_{10}H_{18}O_6$ requires C, 42.55; H, 6.4%; mol.wt. 282). Complete acid hydrolysis yielded only xylose, and partial hydrolysis no sugars other than xylose and the starting material.

Fraction 4. This fraction, which did not crystallize, was a mixture of two substances. The minor component, which had R_x 0.14 in solvent mixture B, and M_n 0.02, was in-

distinguishable from authentic xylotriose. The major component was indistinguishable chromatographically from the trisaccharide found in fraction 5 (see below), but was differentiated from this substance by its higher electrophoretic mobility, the M_n value being 0.5. This fraction has not been examined further.

Fraction 5. This material, originally a colourless syrup, homogeneous both chromatographically and electrophoretically, crystallized on storage. Recrystallization from 85% ethanol yielded the *trisaccharide* as clusters of needles, m.p. 225° decomp., $[\alpha]_D^{20} - 52^\circ \rightarrow -47 \pm 1^\circ$ in 1 hr. in water (c, 3.5); R_x values: 0.72, 0.26, 0.20; M_n value: 0.2 (Found: C, 43.2; H, 6.5; mol.wt. 408, 408. $C_{15}H_{28}O_{13}$ requires C, 43.5; H, 6.3%; mol.wt. 414).

Fraction 6. This material, apparently a tetrasaccharide, was chromatographically homogeneous but could not be made to crystallize. The tetrasaccharide had $[\alpha]_D^{20} - 56.7 \pm 1^\circ$ in water (c, 4.3); R_x values: 0.63, 0.08, 0.06; M_n value: about 0.02 (Found: mol.wt., 526, 538. Calc. for $C_{20}H_{34}O_{17}$: 546).

Examination of the new trisaccharide

Periodate oxidation. The trisaccharide (1.95 mg.) was submitted to oxidation by sodium periodate. Oxidation was complete in 24 hr., at which time the CO_2 liberated from solution corresponded to 2.85 mol.prop. of formic acid, and the periodate consumed was 4.04 mol.prop. These figures are the means of three determinations. Xylose (1.81 mg., equivalent to 85% of the theoretical value for one mol.prop.) was the only sugar found in the hydrolysate of the product from the three combined oxidations.

Decomposition of the trisaccharide by ion-exchange resin. The trisaccharide (17 mg.) was heated in boiling water for 30 min. with hydrated sodium acetate (54 mg.), phenylhydrazine hydrochloride (30 mg.) and water (0.2 ml.). The precipitate of fine orange needles which separated on chilling was collected by centrifuging and dissolved in ethanol. This solution was treated with Zeo-Karb 215 as described by Finan & O'Colla (1955). More satisfactory chromatograms of the sugar in the eluate from the resin were obtained by first evaporating the solution to dryness in

vacuo, extracting the residue with water, and passing through a small column (20 mm. $\times$ 8 mm.) of charcoal-Celite. When this column was washed with increasing concentrations of ethanol in water, only the 5% ethanol eluted any sugar. The substance had R_x values: 1.05, 0.77, 0.67, indistinguishable from rhodymenabiose.

Acid hydrolysis. Complete hydrolysis (2 hr.) yielded only xylose. Partial hydrolysis (0.5 hr.) yielded, in addition to the starting material and xylose, two other substances, R_x (in solvent mixture B) 0.38 and 0.77, the former being the stronger. These corresponded to xylobiose and rhodymenabiose respectively, when run on the same paper.

Examination of the new tetrasaccharide

Periodate oxidation. Oxidation of the tetrasaccharide (2.29 mg.) under the conditions described was complete in 27 hr., when formic acid production and periodate consumption were respectively 2.86 and 4.95 mol.prop. (means of two determinations). Xylose (0.91 mg., equivalent to 72% of the theoretical value for 1 mol.prop.) was the only sugar found in the hydrolysate of the oxidation product.

Decomposition of the tetraosazone by ion-exchange resin. The tetrasaccharide was converted into the osazone as described above. As nothing separated when the reaction mixture was cooled, it was diluted with ethanol and the whole transferred to the Zeo-Karb 215 column. When the decomposition product was placed on a small charcoal-Celite column it was not eluted by water or 5% (v/v) ethanol, but was eluted by 15% (v/v) ethanol. It had R_x values 0.7, 0.24, 0.22, and M_x value 0.5.

Acid hydrolysis. Complete hydrolysis yielded only xylose, whereas partial hydrolysis yielded three substances in addition to unchanged starting material and xylose. In order of decreasing yield, they had R_x values (in solvent B only) 0.39, 0.75 and 0.21, indistinguishable respectively from xylobiose, rhodymenabiose and the trisaccharide found in fraction 5.

Acid hydrolysis of rhodymenan

In Fig. 1, X is shown a chromatogram of a partial acid hydrolysate of rhodymenan (1 hr.). Comparison with the result of a corresponding hydrolysis by rumen bacteria (Fig. 1, Y) shows that, at least for the lower oligosaccharides, the same substances are present in both, although in different proportions. Thus whereas the acid hydrolysate contains more xylotriose than the trisaccharide found in fraction 5, the positions are reversed in the bacterial hydrolysate. Similarly, the acid hydrolysate contains a lesser proportion of the tetrasaccharide found in fraction 6 than does the bacterial hydrolysate.

DISCUSSION

Of the two types of linkage in the wheat-flour pentosan molecule, that between arabinose and xylose seems to be more easily broken by the suspension of rumen bacteria than that between xylose and xylose. For this reason, no mixed arabinose and xylose oligosaccharides were found in the di- and tri-saccharide fractions, and only traces in the tetra- and penta-saccharide fractions. The ease with which the arabinose side chains are split-off by the bacterial suspension parallels the lability of the

arabofuranoside linkage to acid hydrolysis. Perlin (1951b) found that more than three-quarters of the arabinose in the pentosan could be removed under mild acid conditions, which left the xylan chain almost intact. Decomposition of the xylan chain by the rumen bacteria proceeds by apparently random hydrolysis, and not by stepwise attack at the ends of the chain, as is shown by the presence in a partial hydrolysate of a series of higher oligosaccharides (Table 1).

The xylose oligosaccharides obtained from the wheat-flour pentosan in the present work belong to the homologous β -1:4 series. They were first isolated by Whistler & Tu (1951, 1952, 1953) from a partial acid hydrolysate of maize-cob xylan, and later from wheat-straw hemicellulose by Bishop (1955). No oligosaccharides have hitherto been reported from hydrolysates of wheat-flour pentosan.

The chief feature of interest in the experiments on rhodymenan is the production of a number of new xylose oligosaccharides during the hydrolysis by rumen bacteria. These substances were absent from similar hydrolysates of the wheat-flour pentosan (Fig. 1, Y, Z), in which only known substances have so far been detected. It may be noted, before proceeding to consider the new oligosaccharides, that, in addition to xylose itself, xylobiose and xylotriose are present in the hydrolysate of rhodymenan. The presence of xylotetraose and higher members of the β -1:4 series has not yet been established.

The absence of any synthesis when the bacterial suspension was incubated with arabinose, xylose or xylobiose seems to exclude the possibility that these new oligosaccharides arose by transglycosylation. The occurrence of the new xylose oligosaccharides in a dilute solution of rhodymenan after partial acid hydrolysis (Fig. 1, X) is further evidence that they are genuine fragments of the polysaccharide, and are not products of enzymic synthesis. Since the polysaccharide possesses the unique feature of having xylose β -1:3 linkages, these new oligosaccharides must be distinguished from those of the β -1:4 series by their having one or more β -1:3 linkages.

Rhodymenabiose. The low yield of this substance from the hydrolysate of rhodymenan has not allowed a complete investigation of its chemical properties. The formation of xylose only on complete hydrolysis, and the molecular weight as determined by hypiodite oxidation, show the substance to be a xylose disaccharide. In agreement with this is the observation that during hydrolysis by dilute acid no sugars are detectable chromatographically other than rhodymenabiose and xylose. In the light of what is known of the structure of the parent polysaccharide, rhodymenabiose must therefore be *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-D-xylopyranose. An interesting feature of

the substance is its high mobility on the paper chromatogram. In solvent mixtures *B* and *C* it is twice as mobile as xylobiose ($R_{xylobiose}$ values, 1.97, 1.98 respectively), and in solvent mixture *A* it is more mobile than xylose itself (R_x value, 1.05). This is the first instance known to the author of a disaccharide having a higher chromatographic mobility than the parent monosaccharide.

The trisaccharide. This substance was obtained in approximately 5% yield from the xylan. Elementary analysis and hypiodite oxidation showed it to be a trisaccharide, $C_{18}H_{26}O_{13}$. Assuming only that the molecule consists of three β -xylopyranose units, it is possible from the results of periodate oxidation and other experiments to assign a structure without reference to the parent polysaccharide. One mole of the substance, when oxidized by periodate, consumed four moles of oxidant, yielding three moles of formic acid, and one mole of xylose remained intact in the oxidation product. Of the 12 possible trisaccharides, only *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose (3²- β -xylosylxylobiose) would be expected to give these proportions on oxidation. Confirmation that the new trisaccharide has this structure is provided by the results of partial hydrolysis, both of the substance itself by dilute acid, and of the osazone by ion-exchange resin. In the former case two intermediate products of hydrolysis are detectable in addition to xylose, indistinguishable chromatographically from xylobiose and rhodymenabiose. Finan & O'Colla (1955) examined the effect of an acidic ion-exchange resin under controlled conditions on osazones of oligosaccharides. In each case, only the glycosidic linkage nearest to the osazone grouping was hydrolysed, yielding the osazone of a monosaccharide and a reducing sugar containing one monosaccharide unit less than the original oligosaccharide. Applying this technique to the trisaccharide, the substance formed by action of the resin was chromatographically indistinguishable from rhodymenabiose. The results of these two hydrolytic experiments are explicable only if the trisaccharide is 3²- β -xylosylxylobiose, confirming the conclusion drawn from the periodate oxidation. The structure advanced for the trisaccharide is, of course, in agreement with what is already known of the structure of rhodymenan.

3²- β -Xylosylxylobiose resembles rhodymenabiose in being much more mobile on a paper chromatogram than the corresponding oligosaccharide containing only 1:4 linkages, the $R_{xylobiose}$ values being 1.36, 1.94 and 1.43 respectively in the three solvent mixtures.

The tetrasaccharide. This material, although chromatographically pure, has not yet been obtained in crystalline form. The complete hydrolysis

of the material yielding only xylose, and the molecular weight as determined by hypiodite oxidation, show it to be a xylose tetrasaccharide.

Periodate oxidation of the tetrasaccharide yielded on a molar basis 3 moles of formic acid, consumed 5 moles of periodate, and left 1 mole of xylose intact in the oxidized product. Of the eight possible linear β -xylopyranose tetrasaccharides containing only 1:3 or 1:4 linkages or both, only two would be expected to give these proportions on oxidation. These are (a) *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose, i.e. 3²- β -xylobiosylxylobiose, and (b) *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose, i.e. 3³- β -xylosylxylotriose. A choice between these cannot be made with certainty on the evidence available at present. Structure (a), however, seems rather more probable, for the following two reasons. (1) Xylotriose is apparently not formed during partial hydrolysis of the tetrasaccharide. (2) Hydrolysis of the osazones of (a) and (b) according to the procedure of Finan & O'Colla (1955) should yield respectively 4²- β -xylosylrhodymenabiose and 3²- β -xylosylxylobiose, i.e. the trisaccharide already found in the bacterial hydrolysate of rhodymenan. The product formed in this reaction from the osazone of the tetrasaccharide cannot be 3²- β -xylosylxylobiose, because of the widely differing electrophoretic mobilities of the two substances.

Like rhodymenabiose and the new trisaccharide, the tetrasaccharide has a higher chromatographic mobility than the corresponding oligosaccharide containing only 1:4 linkages; the $R_{xylobiose}$ values in the three solvent mixtures are 2.2, 2.0 and 2.1.

When $\log(1/R_F - 1)$ (Bate-Smith & Westall, 1950) of the oligosaccharides present in the partial hydrolysate of rhodymenan, calculated from a chromatogram in solvent *B*, is plotted against the degree of polymerization, the points fall on two nearly parallel straight lines (Fig. 2). The upper line represents the oligosaccharides containing only 1:4 linkages, the lower one those oligosaccharides containing one 1:3 linkage. It is also of interest to note that rhodymenabiose and the new trisaccharide are more firmly held on a charcoal column than are the corresponding oligosaccharides containing only 1:4 linkages.

The value of establishing structures of oligosaccharides as a means to understanding the nature of the polysaccharides from which they are produced has become widely recognized recently (see, for example, Hirst, 1955). The present investigation therefore might possibly shed light on the structure of rhodymenan. The isolation in substantial yield of a pure crystalline trisaccharide

Fig. 2. Relationship between chromatographic mobility and degree of polymerization of xylose oligosaccharides. O, β -1:4 series; Δ , oligosaccharides from rhodymenan, each containing one β -1:3 linkage. Solvent mixture B. R_F calculated from R_F multiplied by R_F of xylose (0.31).

containing both a 1:3 and a 1:4 linkage affords conclusive proof that the polysaccharide molecule does indeed contain both types of linkage. This conclusion had been previously drawn from the failure of all attempts to separate the xylan into an all 1:4 fraction and an all 1:3 fraction. No substances were detected in the hydrolysate which could be the trisaccharide 3²- β -xylosylrhodymenabiose or the tetrasaccharides 3²- β -xylobiosylrhodymenabiose or 3²- β -rhodymenabiosylxylobiose. The conclusion may thus be tentatively drawn that the rhodymenan chain does not contain two adjacent 1:3 linkages.

In contrast to the hydrolysis of the wheat-flour pentosan, which proceeds in a similar fashion whether carried out by acid or by the rumen bacteria, the hydrolysis of rhodymenan by these two agencies shows some qualitative differences (Fig. 1, X and Y). The total proportion of substances containing the 1:3 linkage (spots c, f and h) appears to be greater in the bacterial hydrolysate, suggesting that the relative susceptibility to hydrolysis of the two types of linkage varies according to the hydrolytic agent. Duncan, Manners & Ross (1954, 1956) suggested that when attacked by the enzymes of *R. palmata* itself, rhodymenan splits rather more readily at the 1:3 linkage.

SUMMARY

1. The hydrolysis of two water-soluble pentosans by toluene-treated suspensions of bacteria from sheep rumen has been studied.

2. The pentosan of wheat flour and the xylan of the red seaweed *Rhodymenia palmata*, for which the trivial name rhodymenan is proposed, are both hydrolysed to completion by the bacterial suspension under physiological conditions.

3. Hydrolysis of the wheat-flour pentosan by the bacterial suspension proceeds by removal of the arabinose side chains and fragmentation of the main xylose chain simultaneously. Partially hydrolysed pentosan was fractionated on a charcoal column, yielding arabinose and xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, together with a series of higher oligosaccharides. Oligosaccharides of low molecular weight, containing both arabinose and xylose, are present in the hydrolysate only in traces.

4. Rhodymenan, when partially hydrolysed by the bacterial suspension, yields xylose, xylobiose and xylotriose, together with a number of hitherto unknown xylose oligosaccharides. Two of the latter have been obtained in crystalline form; one is a disaccharide for which the name *rhodymenabiose* is proposed and the other is a trisaccharide. An amorphous xylose tetrasaccharide has also been isolated.

5. The structure *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-D-xylopyranose is suggested for rhodymenabiose. The structure of the trisaccharide has been shown to be *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose. The sequence of linkages in the tetrasaccharide is either β -1:4, β -1:3, β -1:4 or, less probably, β -1:3, β -1:4, β -1:4.

6. The structure of rhodymenan is discussed in the light of these findings.

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The Purification and Properties of Rabbit-Muscle Myokinase

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Previous methods for purifying myokinase (adenylate kinase) have made use of the procedure of Colowick & Kalckar (1943), which has been improved by Noda & Kuby (1955, 1956), Bendall (1954) and Bowen & Kerwin (1955). A different method, to be described here, yields a preparation which appears to be very similar in its physical properties to the crystalline enzyme of Noda & Kuby (1956). A study has also been made of the properties of the sulphydryl groups of the enzyme and of its heat stability.

Preliminary reports of this work have been given elsewhere (Callaghan, 1956, 1957).

EXPERIMENTAL

Materials and methods

Adenosine triphosphate (ATP). This was obtained as the disodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Adenosine diphosphate (ADP). This was prepared by the dephosphorylation of ATP by means of the potato adenylyl pyrophosphatase of Lee & Eiler (1951), and the barium salt was prepared according to Le Page (1951). This was stored at -15° . Chromatographic analysis indicated that 94.5% of the phosphate was present in the form of ADP, 1% as adenosine 5'-monophosphate (AMP) and 4.5% as inorganic phosphate.

Adenosine 5'-monophosphate. AMP was obtained from Roche Products Ltd., Welwyn Garden City, England. Solutions of AMP were brought to pH 7 before use.

Sodium creatine phosphate hexahydrate. This was a gift from the Sigma Chemical Co.

L-Cysteine hydrochloride. The preparation (British Drug Houses, London) was dissolved in water and carefully neutralized to pH 7.

Sodium p-chloromercuribenzoate. This was a gift from Dr G. Weber.

Methylmercury nitrate. This was prepared from methylmercuric iodide by treatment with silver nitrate in ethanol (Johns, Peterson & Hixon, 1930). The product was twice recrystallized from water, and had a melting point of $59-60^{\circ}$. Methylmercury iodide was prepared from mercury and methyl iodide by the photochemical method of Maynard (1932), and, after two crystallizations from alcohol, had a melting point of 144° . The combined methods gave a yield of 70% of methylmercury nitrate.

Haemoglobin and cytochrome c. These were gifts of Dr F. W. J. Teale.

Hexokinase and glucose 6-phosphate dehydrogenase. A mixture of these enzymes ('coupling enzyme') was obtained from yeast by the method of Oliver (1955). The freeze-dried preparation was stored in the refrigerator.

Crystalline bovine-plasma albumin. This was obtained from Armour Laboratories, Lindsey St., London, E.C. 1.

Spectrophotometer. The Unicam spectrophotometer model SP. 500 was employed for the determination of optical density. At 340 m μ , glass cuvettes (1 cm.) were used and quartz cuvettes (1 cm.) were used to measure optical density at 260 and 280 m μ .

In assaying enzyme activity, the temperature of the cuvettes was controlled by a water-jacketed cell compartment made according to Campbell & Simpson (1953).

Routine assay of myokinase. The enzyme was assayed by the method of Oliver (1955). This method gave duplicate results which agreed within $\pm 5\%$ of each other, and was invaluable for the rapid assay of the enzyme in any large series of experiments. The method depends on the reactions: