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The reaction between epichlorohydrin and polysaccharides ☆: structural elements in a cross-linked dextran, Sephadex® G-25

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Abstract

Different types of structural elements in Sephadex® G-25, a dextran that has been cross-linked by reaction with epichlorohydrin, have been characterised. The method used involved hydrolysis with acid, reduction with sodium borodeuteride, permethylation, and investigation of the product by GLC and GLC-MS. Several structural elements were identified, and only a minor part of these were involved in cross-linkages.

Keywords: Cross-linked polysaccharides; Sephadex; Epichlorohydrin

1. Introduction

In our studies of the reaction between epichlorohydrin and polysaccharides, we have synthesised derivatives of methyl α-D-glucopyranoside, representative of different types of possible structural elements in cross-linked dextran [1,2]. We now report studies of the structural elements in Sephadex® G-25, a commercial product prepared from the

☆ Part 3. For Part 2, see ref [2]. Taken from the PhD thesis of L.H., Swedish University of Agricultural Sciences, Uppsala, 1983.

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dextran elaborated by *Leuconostoc mesenteroides* NRRL B512, by reaction with racemic epichlorohydrin in aqueous 10% sodium hydroxide. This dextran is an essentially (1 → 6)-linked α-D-glucan with 5% branching points, mainly through 3-positions. There are indications that most of the branches are short, containing one or two α-D-glucopyranosyl residues [3].

2. Results and discussion

Hydrolysis of the glycosidic linkages in Sephadex® G-25 was performed in two steps, first with 90% formic acid at 100°C for 2.5 h, and then with 3 M sulfuric acid at 100°C for 5 h. These conditions are stronger than those needed for complete hydrolysis of dextran, probably because the glycosidic residues in which a 1,4-dioxane ring is fused to the pyranosyl ring (see below) are more resistant than the others.

The amount of unsubstituted glucopyranosyl residues in Sephadex® G-25 was determined by two methods. A sugar analysis of a hydrolysate, as the alditol acetates, by GLC and with D-mannose as an internal standard, gave 47% unsubstituted residues. On periodate oxidation, formic acid is formed from the unsubstituted residues, and the amount corresponded to 42% of such residues. Since the 3,6-disubstituted residues (~ 5%) are not oxidised, the agreement between these analyses is good.

The total amount of dextran in Sephadex® G-25 was determined to be ~ 80% by reduction of a hydrolysate with tritiated sodium borohydride and determination of the amount of tritium incorporated in the product (unpublished results). As ~ 6 mol% internal glycosides are formed during the hydrolysis (see below) the real value should be somewhat higher, ~ 85%. From these values, the molar percentage of unsubstituted glucopyranosyl residues in Sephadex® G-25 is estimated to be ~ 55%.

The hydrolysate was reduced with sodium borodeuteride, permethylated, and analysed by GLC (Fig. 1). The components were investigated by GLC-MS, using chemical ionisation in ammonia for determination of molecular weights, and electron impact for further characterisation. The spectra were also compared with those of authentic materials, prepared by similar treatment of the model substances. The typical fragmentation patterns have been discussed in connection with these studies [1,2].

The component in peak 1 (M = 267) was identified as D-glucitol-*1-d* hexamethyl ether, derived from unsubstituted glucopyranosyl residues (structural element A). The different types of structural elements are summarised in Scheme 1.

The components in peaks 2–4, 6, 8, and 10 all had M = 309. Four of these (2,4,8,10), from their EI mass spectra, are derived from glucopyranosyl residues in which a 1,4-dioxane ring is fused to the 2-and 3-positions. Those in peaks 2 and 4 were indistinguishable from the corresponding derivatives prepared from methyl 2,3-*O*-[(2*R*)-3-hydroxypropane-1,2-diyl]-α-D-glucopyranoside and its (2*S*)-isomer [2], respectively. These are the only components derived from substituted glucopyranosyl residues for which the structures were fully defined. The components in 8 and 10 are consequently derived from the two corresponding 2,3-*O*-(1-hydroxypropane-2,3-diyl)-α-D-glucopyranosyl residues. The components in 3 and 6, and part of that in 2, had mass spectra similar to those of the permethylated alditols prepared from methyl 3,4-*O*-(3-hydroxy-

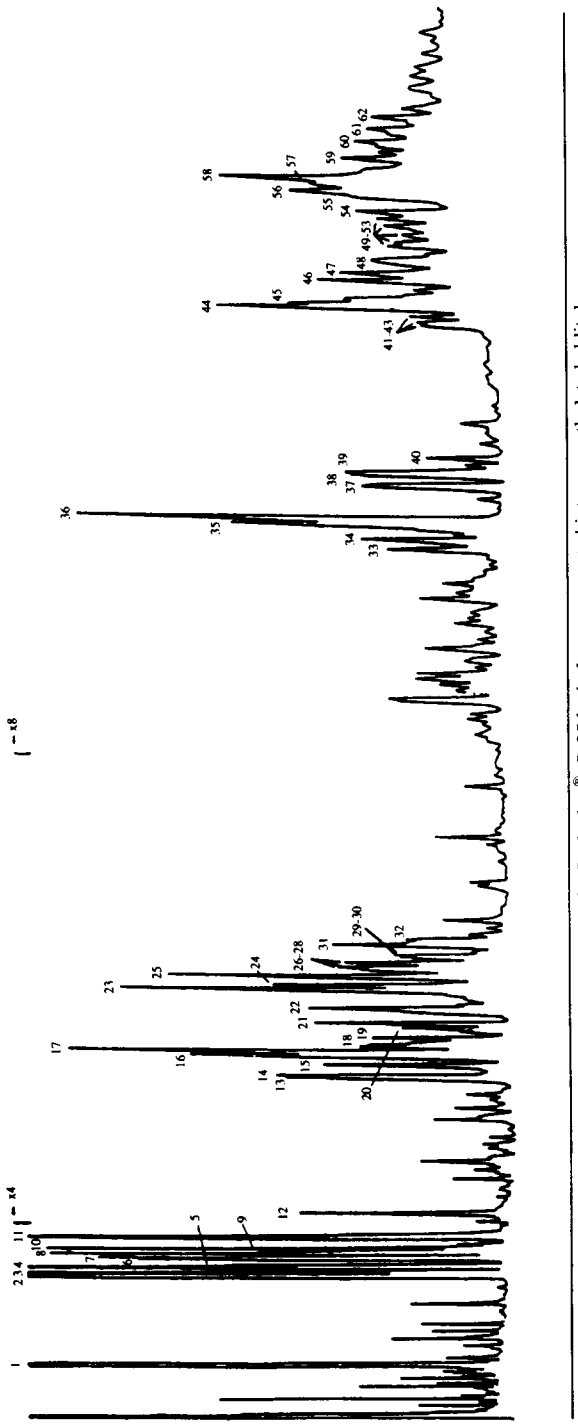
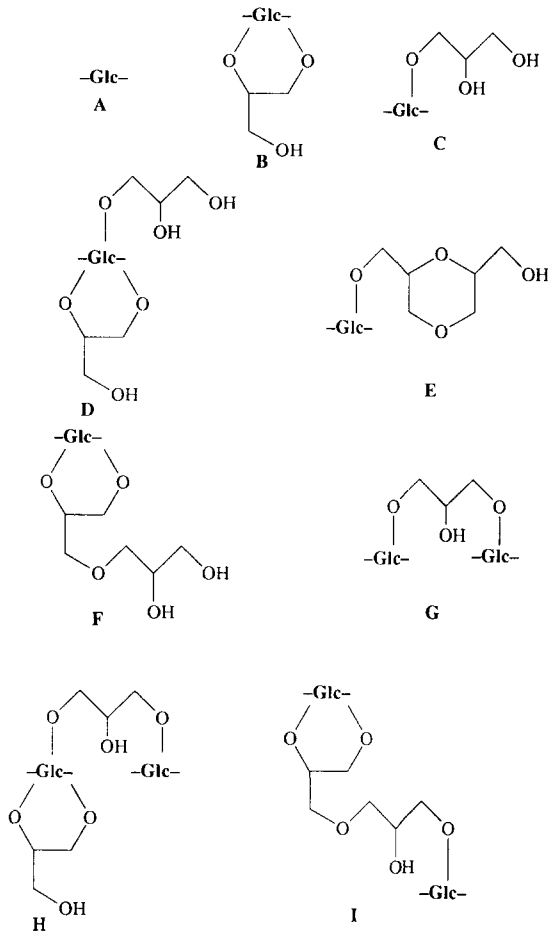


Fig. 1. Gas chromatogram of a Sephadex® G-25 hydrolysate converted into permethylated alditols.



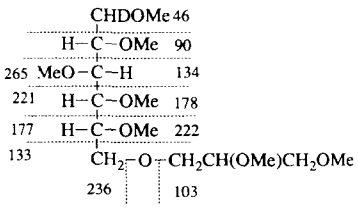
Scheme 1. Structural elements in Sephadex® G-25.

propane-1,2-diyl)-α-D-glucopyranoside [2], and thus arise from glucopyranosyl residues in which the substituent is fused to the 3- and 4-positions. All these substances consequently arise from glucopyranosyl residues with the general structure B.

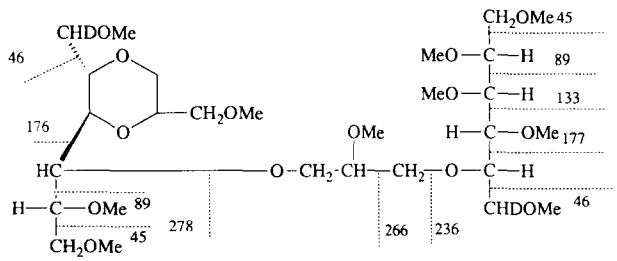
The components in three peaks, 5, 7, and 9, had M = 292, demonstrating that they are internal glycosides. Such glycosides are also formed on acid hydrolysis of methyl 2-*O*-(2,3-dihydroxypropyl)-α-D-glucopyranoside [1], and the components arise from 2-*O*-(2,3-dihydroxypropyl)-α-D-glucopyranosyl residues. As before, no attempts were made to identify the individual components. Peak 11 (M = 355), from its EI mass spectrum, is a mixture of permethylated *O*-(2,3-dihydroxypropyl)-D-glucitol derivatives. The corresponding substances prepared from the methyl 2-, 3-, and 4-*O*-(2,3-dihydroxypropyl)-α-D-glucopyranosides [1] were also not separated on the column used. The presence of the 3-substituted derivative cannot be demonstrated by mass spectrometry

when it is mixed with the 2- and 4-substituted derivatives, but the mixture most probably contains all three components, derived from 2-, 3-, and 4-*O*-(2,3-dihydroxypropyl)-α-D-glucopyranosyl residues. They thus arise from the general type C, in which a glucopyranosyl residue carries an *O*-(2,3-dihydroxypropyl) substituent.

The component in peak 12, also with M = 355, from its EI mass spectrum, arises from a 6-*O*-(2,3-dihydroxypropyl)-α-D-glucopyranosyl group. The origin of some primary fragments is indicated in the formula. Although a terminal glucopyranosyl group should be at least as reactive as a chain residue, this is the only component observed for which the origin from such a group is demonstrated.



12 M=355



44, 46 M=618

Components 13–17 all had M = 397. The first two had the same mass spectra and retention times as the two components obtained from methyl 4-*O*-(2,3-dihydroxypropyl)-2,3-*O*-[(2*R*)-3-hydroxypropane-1,2-diyl]-α-D-glucopyranoside [2]. Compounds 15–17 gave similar mass spectra and are isomers, also containing a 2,3-fused substituent. The mass spectra of all these substances contain *m*/*z* 293 and 261, formed by consecutive eliminations of the cyclic substituent and methanol, as previously discussed [2]. These substances are derived from the general type D.

The component in peak 22 was too weak to give a good mass spectrum, but had the same retention time as one of the products obtained from methyl 2-*O*-(6-hydroxymethyl-1,4-dioxan-2-ylmethyl)-α-D-glucopyranoside [2]. It therefore possibly arises from a structural element of the general type E, an *O*-(6-hydroxymethyl-1,4-dioxan-2-ylmethyl)-α-D-glucopyranosyl residue. This type does not seem to be very abundant.

The component in peak 23, with M = 397, had the same retention time and mass spectrum as that derived from methyl 2,3-*O*-[(2*R*)-6,7-dihydroxy-4-oxaheptane-1,2-

diyl]-α-D-glucopyranoside [2]. The component in peak 25 had a similar mass spectrum and arises from a related structure. The mass spectra contain the typical ions m/z 205 and 173, formed by consecutive eliminations of the cyclic substituent and methanol. The substances arise from a structural element of the general type F.

The components in peaks 35 and 36, with M = 513, had the same retention times and mass spectra as two products obtained from 2-hydroxy-1,3-bis(methyl α-D-glucopyranosid-2-*O*-yl)propane [1]. They are internal glucosides, and are derived from a structural element of the general type G, representing a cross-linkage.

The components in peaks 44 and 46, of M = 618, according to their mass spectra, arise from a structural element in which a type D element is linked to the 2-position of a glucopyranosyl residue, and belongs to the general type H. A suitable model substance was not available. In addition to the ions indicated in the formula, they gave the typical ion m/z 482, formed by consecutive eliminations of the cyclic substituent and methanol.

The components in peaks 56 and 58, with M = 555, had the same mass spectra as two components obtained from the model substance methyl 2,3-*O*-[(2*R*)-6-hydroxy-7-(methyl α-D-glucopyranosid-2-*O*-yl)-4-oxaheptane-1,2-diyl]-α-D-glucopyranoside [2]. The components are internal glucosides and, in additon to the ions indicated in the formula, gave m/z 205 and 173 by consecutive eliminations of the cyclic substituent and methanol. They consequently arise from structural elements of type I. In the general type I, a residue of type B and an α-D-glucopyranosyl residue are connected via a 2-hydroxy-1,3-propanediyl group, linked to the latter and to the substituent of the α-D-glucopyranosyl residue in B.

The different types of structural elements are summarised in Scheme 1. The peaks in the gas chromatogram (Fig. 1) are gathered into five well-separated regions, the first of which (1) consists of a single peak, derived from unsubstituted glucopyranosyl residues (type A). The peaks in the second region (2–12) arise from glucopyranosyl residues substituted with one C_3 residue (types B and C). The third region contains peaks deriving from glucopyranosyl residues substituted with two C_3 residues (types D, E, and F). The fourth region contains peaks derived from two cross-linked glucopyranosyl residues and one C_3 residue (type G). The fifth region, finally, contains peaks derived from two cross-linked glucopyranosyl residues and two C_3 residues (types H and I). Most of the peaks in the three last regions gave weak mass spectra and the components in them could not be definitely identified. It seems most probable, however, that they also arise from structural units in which the proportions of glucopyranosyl to C_3 residues are 1:2, 2:1, and 2:2, respectively. In order to determine the molar proportions of the components in the five regions, effective carbon relative response factors for the flame-ionisation detector [4] were calculated for the identified products. For components with the same proportions of glucopyranosyl to C_3 residues but of different types, the calculated response factors should be the same or closely similar, and the errors introduced by using the same factor for all non-identified compounds in a region should be insignificant. The results are given in Table 1.

Even if types A–I are the most abundant structural elements in Sephadex® G-25, it is obvious that it also contains other structural elements. Thus, there are other alternatives for the elements containing glucopyranosyl and C_3 residues in the proportions 1:2 and

Table 1
GLC of hydrolysed, reduced, and permethylated Sephadex® G-25

Peak number	Area	Glc:C_3	Structural element	Response factor [a]	Mol% [b]
1	100	1:0	A	0.60	55.70
2	15.0	1:1	B	0.70	7.15
3	6.9	1:1	B	0.70	3.31
4	9.2	1:1	B	0.70	4.48
5 [c]	1.7	1:1	C	0.60	0.94
6	5.0	1:1	B	0.70	2.37
7 [c]	3.9	1:1	C	0.60	2.17
8	5.4	1:1	B	0.70	2.67
9 [c]	2.0	1:1	C	0.60	1.10
10	4.7	1:1	B	0.70	2.24
11	11.2	1:1	C	0.80	4.68
12	0.6	1:1	C	0.80	0.27
13	0.8	1:2	D	0.90	0.30
14	0.7	1:2	D	0.90	0.27
15	0.7	1:2	D	0.90	0.27
16	1.4	1:2	D	0.90	0.53
17	1.8	1:2	D	0.90	0.67
18–21	2.1	1:2	—	0.90	0.77
22	1.5	1:2	E	0.90	0.57
23	2.1	1:2	F	0.90	0.77
24	1.3	1:2	—	0.90	0.47
25	1.8	1:2	F	0.90	0.67
26–32	4.7	1:2	—	0.90	1.77
33–34	0.8	2:1	—	0.55	0.50
35 [c]	0.8	2:1	G	0.55	0.50
36 [c]	1.1	2:1	G	0.55	0.67
37–40	1.5	2:1	—	0.55	0.90
41–43	0.8	2:2	—	0.65	0.40
44	0.7	2:2	H	0.70	0.33
45	0.4	2:2	—	0.65	0.20
46	0.4	2:2	H	0.70	0.20
47–55	1.8	2:2	—	0.65	0.94
56 [c]	0.8	2:2	I	0.60	0.43
57	0.3	2:2	—	0.65	0.17
58 [c]	0.7	2:2	I	0.60	0.40
59–60	0.4	2:2	—	0.65	0.20

[a] Response factor per glucopyranosyl residue. [b] Molar percentage of glucopyranosyl residues. [c] Internal glucoside.

2:2, and elements with a higher proportion of C_3 residues are certainly present in low percentages. In the identified structural elements, C_3 residues in non-cyclic structures are linked through primary positions, which are the most reactive, and those in cyclic structures to adjacent positions, which gives 6-membered rings instead of the less favoured 7-membered rings. The alternative modes of linkages, although less favourable, are however not excluded.

The results of the investigation are summarised in Table 2. The percentage of

Table 2
Structural elements found in Sephadex® G-25

Glc:C_3	Type	Mol% [a]
1:0	A	55.7
1:1	B	22.1
1:1	C	9.2
1:2	D [b]	2.8
1:2	E [b]	0.6
1:2	F [b]	3.7
2:1	G [c]	2.6
2:2	H,I [d]	3.3

[a] Molar percentage of glucopyranosyl residues. [b] Some components deriving from the 1:2 category of structural elements were not characterised, and the molar percentages of the different types within this category are therefore not very accurate. [c] Several components in the 2:1 region were not characterised, but all components in this region are derived from type G structural elements. [d] Other types of the 2:2 category of structural elements are possible but have not been specified.

unsubstituted glucopyranosyl residues (~ 55%) agrees well with the value found by direct analysis. Most of the substituted glucopyranosyl residues (types B, D, F, H, and I) (~ 30%) contain a fused 1,4-dioxane ring. Rather few glucopyranosyl residues (~ 6%) are involved in cross-linkages. Higher-substituted glucopyranosyl residues should be present in small amounts but have been overlooked, and the molar percentages of substituted and of cross-linked residues are therefore probably slightly underestimated.

3. Experimental

General methods.—GLC was run on a Hewlett–Packard 5880 A instrument, fitted with a flame-ionisation detector and an OV-1 fused-silica column (25 × 0.32 mm), and using a temperature program from 200–300°C at 1.3°C min^{-1}. For GLC-MS, a Finnigan 4021 instrument equipped with a Finnigan 9610 gas chromatograph was used. Separations were performed on an OV-1 fused-silica column, using the same temperature program as above. EI mass spectra were recorded at 70 eV. For CI mass spectra, ammonia was used as ionisation gas.

Hydrolysis with acid.—A commercial sample of Sephadex® G-25 medium (100 mg) was treated with 90% formic acid (5 mL) at 100°C for 2.5 h, when a clear solution was obtained. The solution was concentrated, the residue dissolved in 3 M H_2SO_4 (10 mL), and the solution kept at 100°C for 5 h. The solution was neutralised with $BaCO_3$, filtered, treated with Dowex 50 (H^+), filtered, and concentrated to dryness. The yield of hydrolysed product was 90 mg. When milder conditions were used, the hydrolysis was incomplete, as demonstrated by GLC of the reduced and methylated product.

Determination of unsubstituted residues in Sephadex® G-25.—Sephadex® G-25 (4.43 mg) was hydrolysed as above in the presence of D-mannose (1.55 mg). The resulting syrup was converted into alditol acetates and analysed by GLC.

In another experiment Sephadex® G-25 (198.5 mg) was treated with 50 mM $NaIO_4$ (50 mL) in the dark at 4°C. Aliquots (0.1 mL) were removed every 24 h and diluted with

water to 100 mL, and the UV absorption was measured. After 6 days, when the consumption of periodate had ceased, a 10-mL sample of the solution was treated with ethylene glycol (1 mL), and formic acid was determined by titration with 10 mM NaOH. The consumpion was 10.34 mL, compared to 0.06 mL for a blank. The total amount of formic acid formed was estimated to be 0.515 mmol.

Methylation analysis of hydrolysed Sephadex® G-25.—The hydrolysed product was reduced with $NaBD_4$. A dried sample of the reduced material (10–20 mg) was dissolved in a mixture of Me_2SO (1 mL) and CH_3I (1 mL), powdered KOH (100 mg) was added, and the mixture stirred at room temperature for 30 min [5]. The mixture was diluted with water (10 mL) and extracted with CH_2Cl_2 (4 × 5 mL), and the combined extract washed with water (4 × 5 mL), concentrated, and analysed by GLC and GLC-MS. The relative areas under the peaks (Table 1), on GLC using a flame-ionisation detector, are average values from four different analyses. The values in the different analyses deviated by less than 1%.

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