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Note

Coupling of proteins and other amines to carbohydrate polymers *via* bromine oxidation and reductive amination

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Polysaccharide polymers, such as cross-linked dextran or agarose derivatives (Sephadex and Sepharose, respectively) and cellulose, are useful solid supports for affinity chromatography[1]. Biologically active compounds can be coupled in alkaline media to polysaccharide polymers that are activated with cyanogen halides[2]. Polysaccharides may also be activated by periodate oxidation[1], as the resulting aldehyde groups can react with amines to give Schiff bases that can be stabilised by reduction[3].

Carbonyl groups can also be generated in polysaccharides by treatment with bromine in aqueous solution[4,5]. When Sephadex, Sepharose, or cellulose were treated with aqueous bromine at pH 7.0, products were formed which became yellow when treated with aqueous sodium hydroxide due to the presence of alkali-labile keto-glycosyl units[6]. Reduction of the bromine-oxidised polymers with sodium borohydride eliminated this property.

Suspensions of the oxidised polysaccharides in water at pH 6.5 or in formamide under neutral conditions reacted with primary amines, and the resulting Schiff bases

TABLE I

REDUCTIVE COUPLING OF AMINES TO BROMINE-OXIDIZED POLYSACCHARIDES

Polysaccharide	*Degree of oxidation* (*Mol of Br_2/mol of polysaccharide*)	*Coupling yields*[a] (*weight %*)			
		1,6-Diaminohexane		*1-Aminodecane*	*Albumin*
Sepharose	0	0[b]	0[c]	0[b]	0[b]
	0.25	1.9[b]	4.8[c]	2.4[b]	6.9[b]
	0.50	3.8[b]	6.3[c]	—	6.8[b]
Sephadex	0	0[b]	—	—	—
	0.20	1.8[b]	—	—	—
	0.40	2.6[b]	—	—	—
Cellulose	0	—	—	—	0[b]
	~0.10	—	—	—	1.0[b]

[a]A tenfold excess of amine was used in the coupling reactions. [b]Coupling performed in water at pH 6.5. [c]Coupling performed in formamide at neutral pH.

were reduced to the corresponding secondary amines with sodium cyanoborohydride[7]. The formation of Schiff bases is favoured by the non-aqueous solvent, but formamide is not always suitable for coupling biologically active substances. The rate of reduction of carbonyl groups in water at pH 6–8 is low compared to that of Schiff bases[7,8]: at pH > 7, the oxidised polysaccharides are degraded[6]. The coupling reactions in water were therefore performed at pH 6.5, and fair yields of products were obtained. Reduction was carried out at room temperature for 3 days to ensure completion of reaction of C=N and C=O groups. The degree of substitution was measured by amino acid analysis[9] or n.m.r. spectroscopy on the partial, acid hydrolysates[10].

Data on the coupling of 1,6-diaminohexane, 1-aminodecane, and albumin are given in Table I.

The method complements existing methods[11] for derivatising polysaccharides.

EXPERIMENTAL

General methods. — N.m.r. spectra were recorded with a Varian HA-100 D spectrometer. G.l.c. was performed with a Varian model 2700 instrument, fitted with a flame-ionisation detector. Separations were performed on glass columns (240 × 0.15 cm) containing 3% of OV 225 on Gas Chrom Q (100–120) mesh at 200°. G.l.c.–m.s. was performed with a Varian CH-7 gas chromatograph–mass spectrometer.

Oxidation of polysaccharides — Sepharose Cl-4B (6 g) and Sephadex G-50 (8 g) were swollen in water for 24 h and then suspended at 30° in 0.1M bromine (100, 200, or 400 ml) adjusted to pH 7.0, and automatically maintained thereat by the addition of 0.5M sodium hydroxide (Metrohm E 300B pH meter). When the oxidant was consumed (4–8 h), the products were washed with water (2 litres), and samples were suspended in water, reduced ($NaBH_4$), neutralised (HOAc), hydrolysed (0.25M H_2SO_4, 95°, 18 h), neutralised ($BaCO_3$), converted into alditol acetates, and subjected to g.l.c. analysis[12].

The formation of glucitol, in addition to 3,6-anhydrogalactitol and galactitol, from modified Sepharose indicated that oxidation had occurred at C-4 of the galactosyl units.

The formation from modified Sephadex of mannitol, allitol, and galactitol, in addition to glucitol, indicated that oxidation had occurred at C-2, C-3, and C-4 in the glucosyl units.

Cellulose (25 g, small pieces of Whatman 3MM paper) was swollen in water for 24 h, and then oxidised with 0.1M bromine (500 ml) as described above. The reaction was slow and only a small amount of the oxidising agent was consumed after 50 h. The product was then collected, washed, and analysed as described above. In this analysis, the formation of mannitol and allitol, in addition to glucitol, indicated that oxidation had occurred at C-2 and C-3 in the glucosyl units.

Coupling reactions. — (*a*) *1,6-Diaminohexane.* To a cold (~5°) solution of 1,6-diaminohexane (5 g) in water (10 ml) adjusted to pH 6.5 with acetic acid, oxidised

Sepharose (0.5 g), or Sephadex (0.5 g), and sodium cyanoborohydride (1 g) were added. The mixtures were stirred at room temperature for 60 h, and the products were collected and washed with 10% aqueous acetic acid (1 litre) and water (1 litre). Samples (100 mg) were hydrolysed in conc. DCl (1 ml) at 100° for 1 h in an n.m.r. tube. The degree of substitution was calculated by comparing the integrated signals of the carbohydrate protons and the methylene protons in 1,6-diaminohexane[10]. The results are given in Table I.

A cold (~5°) solution of 1,6-diaminohexane (5 g) in dry formamide (10 ml) was neutralised with acetic acid, and oxidised Sepharose (0.5 g), prewashed with formamide (4 × 10 ml), and sodium cyanoborohydride (1 g) were added. The mixture was stirred at room temperature for 60 h, and then processed and analysed as described above. The results are given in Table I.

(*b*) *1-Aminodecane.* 1-Aminodecane (7.9 g) was coupled to oxidised Sepharose (0.5 g) suspended in water as described above. The products were washed with 10% acetic acid (1 litre), water (100 ml), 25, 50, 75, and 95% aqueous ethanol (1 litre of each) and finally water (1 litre). The coupling yields were determined as described above, and the results are given in Table I.

(*c*) *Albumin.* Oxidised Sepharose (0.5 g) was suspended in water and added, together with sodium cyanoborohydride (1 g), to a solution of human serum albumin (KABI, Sweden) (1 g) in phosphate buffer (pH 7, 50 ml). The mixture was stirred for 60 h at room temperature, and the product was collected, washed with water (1 litre), 10% aqueous acetic acid, water, 0.1M sodium hydrogen carbonate, M sodium chloride, water, 0.1M sodium acetate buffer (pH 4.5), and water (500 ml of each). The coupling yield was determined by automatic amino-acid analysis[9], and the results are given in Table I.

To a solution of human serum albumin (600 mg) in phosphate buffer (pH 7, 25 ml), oxidised cellulose (4 g) and sodium cyanoborohydride (500 mg) were added. The reaction was performed, and the product was processed and analysed as described above. The results are given in Table I.

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