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STRUCTURAL ANALYSIS OF HEPARIN BY METHYLATION AND G.L.C.–M.S.: PRELIMINARY RESULTS

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ABSTRACT

Heparin is a complex mixture of polysaccharides differing in biological activity and structure, and attempts to relate this activity to structure have suffered, owing to a lack of sufficiently sensitive and specific analytical methods. Application of methylation analysis to determination of the structure of heparin is described. Carboxyl-reduced heparin was converted into its pyridinium salt, this was dissolved in Me_2SO, and free OH and NH groups were methylated with dimethylsulfinyl anion. Sulfate groups were removed by solvolysis, and after dialysis, the polymer was acetylated and depolymerized by acetolysis. The resulting monosaccharides were converted into alditol acetates, which were separated by capillary, gas–liquid chromatography, and identified by both electron impact and chemical ionization mass spectrometry. Seventeen different monosaccharides were identified in the hydrolyzate. All of the expected internal hexosaminyl and glycosyluronic residues were identified. Although several sugars were identified as nonreducing termini, only a hexosamine 6-sulfate was identified as a reducing-terminus sugar. The results indicate that methylation analysis of heparins and other complex, sulfated glycosaminoglycans is feasible.

INTRODUCTION

Methylation analysis, a powerful technique for structural studies of complex carbohydrates, is used to identify the terminal, branch, and internal saccharides, as well as their linkage positions, from the pattern of partially methylated sugars obtained by hydrolysis of the methylated polysaccharide. Using the extremely powerful base, dimethylsulfinyl anion in dimethyl sulfoxide (Me_2SO)[1], a wide variety of Me_2SO-soluble, complex carbohydrates[1–8] has been methylated in a single step, and analyzed by g.l.c. or g.l.c.–m.s. In general, the only limitation in the applica-

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tion of this technique to the analysis of the structural components of polysaccharides appears to be a lack of solubility of certain polysaccharides[9] in Me_2SO.

In this regard, methylation analysis has not been particularly useful in the structural analysis of sulfated glycosaminoglycans. Their insolubility in Me_2SO has heretofore precluded use of the Hakomori method and other techniques, such as dimethyl sulfate in aqueous base, which do not completely methylate such sulfated glycosaminoglycans as heparin, even after repeated methylation[10]. Heparin, which is widely used as an antithrombotic and anticoagulant agent, is perhaps the most complex member of the glycosaminoglycan group of carbohydrates. As usually isolated, heparin is a heterogeneous mixture of structurally different polysaccharide chains, varying over a wide range of degrees of sulfation[11,12], molecular weight[13], relative amounts of glucosyluronic and idosyluronic residues and of 2-sulfamino- and 2-acetamido-hexosyl residues[14].

We now describe preliminary results showing the applicability of methylation analysis to the determination of the structure of heparin. The key to the successful application of this approach is the solubility[15] of pyridinium salts of heparin in Me_2SO, and, as we have found, these salts can be readily methylated by dimethylsulfinyl anion. Following methylation, the polymer was depolymerized by acetolysis, and the resultant monosaccharides were converted into alditol acetates. Capillary g.l.c. was used to effect a high-resolution separation of the mixture, and both electron impact (e.i.) and chemical ionization (c.i.) mass spectrometry (m.s.) were used in order to identify 17 different monosaccharides obtained from heparin. We suggest that methylation analysis of the structure of heparin may now be applied toward establishing the relationships between the structure and activity of this important polysaccharide. Furthermore, this method may find application in the structural analysis of other sulfated polysaccharides.

EXPERIMENTAL

Heparin. — Heparin (sodium salt, beef lung) was obtained from E. J. Boyer, Pharmaceutical Manufacturing Division of the Upjohn Co. (Lot No. 722EH).

Preparation of dimethylsulfinyl anion. — The dimethylsulfinyl anion was prepared according to the method of Sandford and Conrad[2], the only modification being the use of a controlled-atmosphere (anhydrous N_2) chamber (Dri-Lab, Vacuum/Atmospheres Co.) in which to store the freshly distilled Me_2SO and to introduce aliquots of the anion into vials. The anion was stored in sealed vials at −20° until used. Sodium hydride (5 g) and dried, freshly distilled Me_2SO (50 mL) yielded anion (50 mL) which, upon titration of an aqueous solution with HCl, was found to be 1.6M.

Carboxyl reduction. — Carboxyl reduction of the heparin sodium salt was conducted by the method of Taylor and Conrad[16]. Total uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen[17]. A solution of heparin (15.55 mg, 21.44 μmol of uronic acid) in de-ionized water (1.0 mL) was

added to 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (DEC; 42.7 mg) in water (1.0 mL; pH 4.75). This solution was gently stirred for 2 h at room temperature. Aqueous 3M $NaBH_4$ (4 mL) was added dropwise with stirring, and the solution was heated for 2 h at 50°. The mixture was cooled to room temperature, and 1.5M H_2SO_4 was added dropwise to decompose the excess of $NaBH_4$. The solution of carboxyl-reduced heparin (CR-heparin) was dialyzed overnight against de-ionized water.

Formation of heparin pyridinium salt. — The pyridinium salt of heparin was prepared by the method of Nagasawa *et al.*[15]. An aliquot of the CR-heparin dialyzate (9 mL; 3.4 mg of heparin) was treated with Dowex-50 X-8 (H^+) resin (20–50 mesh; Bio-Rad Laboratories) in order to convert the heparin into the free acid. An excess of pyridine was added, and the mixture stirred and then lyophilized to dryness. The residue was further dried over P_2O_5, and stored in the Dri-Lab.

Methylation of carboxyl-reduced heparin. — The methylation reaction was conducted in the Dri-Lab, using glassware and reagents that had been extensively dried and then stored in the Dri-Lab. The dried pyridinium salt was dissolved in anhydrous Me_2SO (5 mL) by stirring with a magnetic stirrer for 10 min; dimethylsulfinyl anion (0.5 mL) was added, the mixture was stirred for 1 h, and methyl iodide (0.5 mL) was added slowly with stirring. After 1 h, all samples were removed from the Dri-Lab, and a stream of dry N_2 was bubbled through the solutions (now at pH 6–7) for 15 min to remove the excess of methyl iodide.

Desulfation of methylated heparin. — Desulfation was accomplished by the method of Nagasawa *et al.*[15]. The solution just obtained was transferred to a tube having a Teflon-lined screw-cap, and water was added to yield a 1:9 H_2O–Me_2SO solution. The tube was heated for 5 h at 80°, and the solution cooled, dialyzed, and lyophilized. The residue was analyzed for the presence of sulfate according to the method of Silvestri *et al.*[18].

Acetolysis–hydrolysis. — Depolymerization and derivatization were performed by the procedure of Stellner *et al.*[7]. To the lyophilized, desulfated polymer was added 0.25M H_2SO_4 in 95% acetic acid (0.6 mL), and the solution was heated overnight at 80° in a Teflon-lined, screw-capped tube. An equal volume of water was then added, and heating was continued for 5 h. The solution was passed through a column of BioRad AG-3 X-4A (acetate) resin in a Pasteur pipet (0.5 cm × 6 cm; 1.2-mL bed-volume). The hydrolyzate was eluted with methanol, and evaporated with N_2.

Reduction of linkage positions. — The residue was dissolved in water (0.4 mL) and $NaBH_4$ (20 mg) was added. After being kept overnight at room temperature, glacial acetic acid was added dropwise to decompose the excess of borohydride. 9:1 Methanol–toluene (3 × 2 mL) was added, and the solution was evaporated.

Acetylation. — Acetic anhydride (1 mL) was added to the residue, and the mixture was heated for 2 h at 100°. Toluene (2.0 mL) was added, and evaporated to dryness with N_2. The residue was dissolved in $CHCl_3$ (1.0 mL, distilled in glass;

Burdick & Jackson), the solution was washed with water (2 × 1.0 mL), and evaporated with N_2, and the sample was analyzed by g.l.c.–m.s.

G.l.c.–m.s. analysis. — Gas–liquid chromatography–mass spectrometry was conducted in a Hewlett–Packard 5985A GC/MS system equipped with an HP 7929 disc-drive and a 2648A graphics terminal. G.l.c. was accomplished in a 25-m, fused silica-glass, capillary column (0.20 mm i.d.) coated with SE-54 (0.1-mm coating, Hewlett–Packard). The carrier gas was ultra-high-purity helium. The column was inserted through the transfer zone, bypassing the jet separator, and terminating at the ion source of the mass spectrometer. To obtain efficient separation, a temperature program was used: 60° initial temperature, holding for 4.0 min, and then increasing to 70° at 5°/min, whereupon the temperature was raised to 300° at 10°/min. The injection port was maintained at 220° while the transfer zone was held at 300° throughout the analysis. The mass spectrometer was tuned by using perfluorotributylamine, and mass spectra were collected in the positive-ion, electron-impact mode at an ionizing voltage of 70 eV and a source temperature of 200°. Chemical-ionization mass spectra were obtained by using methane as the reactant gas at a source pressure of ~133 Pa. An injection volume of 1.0 μL of the sample $CHCl_3$ (2.0 mL) was used for each analysis in the splitless mode.

RESULTS

Preparation of methylated heparin derivatives. — We have observed that the pyridinium salts of both heparin and CR-heparin can be methylated by the proce-

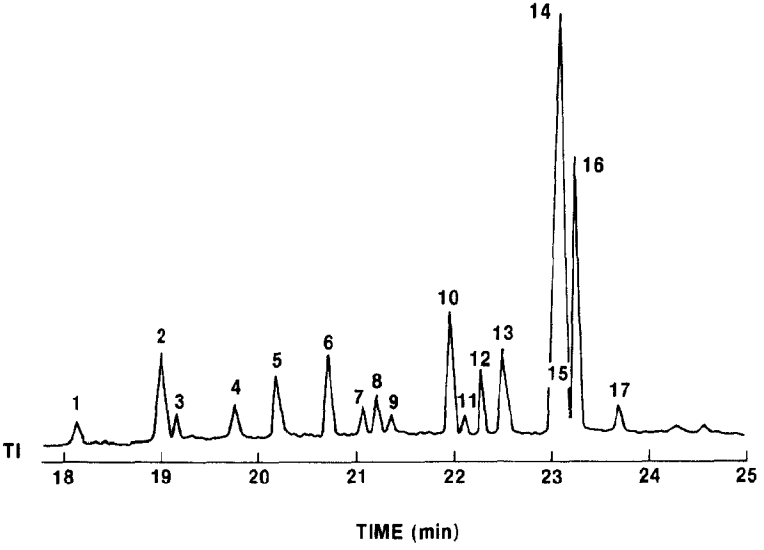


Fig. 1. Total-ion (TI, *m/z*) chromatogram of the compounds obtained from heparin. The identity of these compounds (**1–17**) is presented in Table I. (See Experimental section for reaction and g.l.c. conditions.)

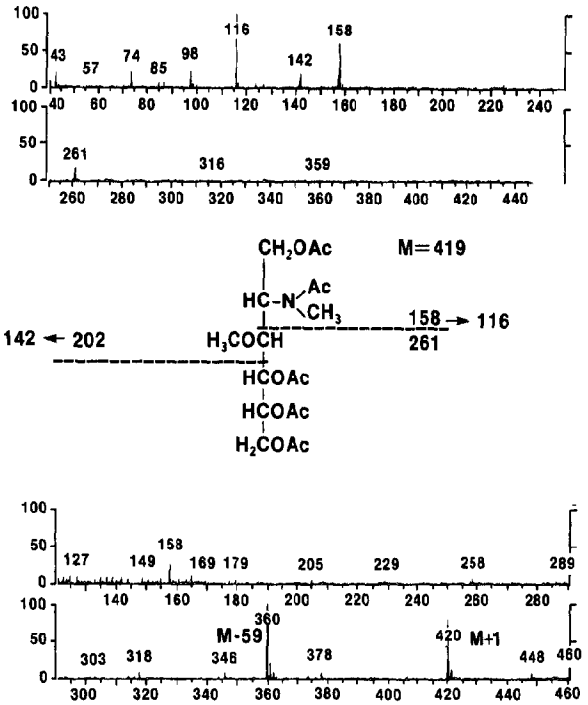


Fig. 2. Electron-impact mass spectrum, proposed fragmentation-pattern, and chemical-ionization (CH_4) mass spectrum, respectively, of the major component, 1,4,5,6-tetra-*O*-acetyl-2-deoxy-3-*O*-methyl-(*N*-methylacetamido)hexitol (compound **14**), obtained in the analysis of heparin components.

dure described. The physical and chemical properties of methylated heparin indicate that the methylation reaction is almost quantitative and does not remove sulfate groups. This is supported by the fact that the product is water-soluble, but not chloroform-soluble, and the molar ratio of sulfate to uronate of the original heparin and of the methylated heparin are both 1.4. Comparison of the infrared spectra (not shown) of pyridinium heparin and methylated pyridinium heparin showed that the broad, OH-stretch band was diminished by >90%, with a concomitant increase in intense CH-stretch bands at 3000–2800 cm^{-1}, with no apparent loss in the bands corresponding to sulfate.

The carboxyl groups were reduced in order to produce derivatives of the uronic acid residues, for which mass spectra have previously been reported[5,7,8]. The initial solution of heparin contained 22.5 μmol of uronate, but, after carboxyl reduction, only 1.6 μmol of uronate remained. If adjusted for the 7% color yield of neutral hexose in the uronate assay[17], it is seen that the reduction step was essentially quantitative.

As already mentioned, the methylation reaction does not desulfate the polymer. However, after desulfation by heating the methylated polymer in Me_2SO–H_2O, no sulfate was detectable. This material was then acetylated, the

acetate depolymerized, the products reduced, the alditols acetylated, and the acetates analyzed by g.l.c.–m.s.

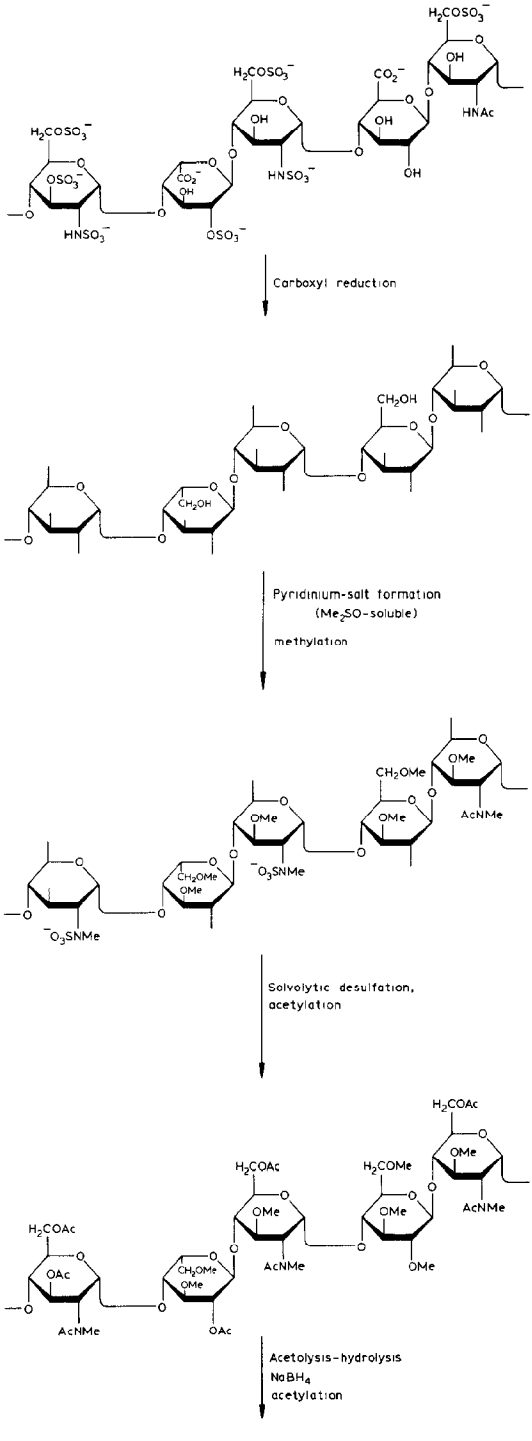
A total-ion chromatogram is shown in Fig. 1. The identification of the sugars, together with a summary of the mass-spectral data obtained from both electron-impact and chemical-ionization analyses are presented in Table I. A total of 17 compounds was identified. Of these, 13 matched compounds having previously published mass spectra, and 12 of the peaks were traceable to sugars previously reported to be present in heparin[19,20]. The e.i. and c.i. mass spectra of the major hexosamine peak are presented in Fig. 2.

DISCUSSION

The derivatization scheme employed in this study (see Scheme 1) was chosen in order to simplify the identification of the monosaccharides formed by producing such derivatives as mixed methyl-, acetyl-hexitols and acetamidohexitols, for which mass-spectral data have previously been published[5,7,8]. In the scheme used in the current study, the methylation reaction labels free OH groups and RNH groups (where R is either acetyl or $-SO_3$). The polymer is then de-*N*- and -*O*-sulfated, and the NHMe and OH groups exposed by this procedure are acetylated, as are the OH groups exposed by depolymerization and reduction. As a consequence, this particular choice of derivatives does not differentiate between *N*-sulfated and *N*-acetylated hexosaminyl residues, as both are converted into the same methylated acetylated derivative. Likewise, positions of sulfation and of glycosidic bonds are not differentiated, because OH groups liberated from both are converted into acetyl derivatives. These are, however, minor shortcomings. The major linkages are well accepted[21,22], and use of different derivatives at selected points in the procedure could eliminate the inability to distinguish between these details of structure.

In determining the identifications shown in Table I, the aforementioned degeneracy must be kept in mind, together with the patterns to be expected from internal and terminal residues. All internal residues will be protected from methylation at both O-1 and O-4, so that these must eventually be acetylated. Nonreducing terminal sugars will become methylated at O-4, but eventually acetylated at O-1, whereas the opposite will be observed for reducing, terminal sugars. Glycosyluronic residues were reduced prior to methylation, so that the resulting hexitols should all have 6-*O*-methyl groups.

Interestingly, although several sugars were identified as nonreducing termini, only hexosamine 6-sulfate residues were identified as reducing-terminal sugars. It has been proposed that the usual heparin fragments of mol. wt. 10,000–20,000 are produced from chains having mol. wt. 100,000 by the action of an endoglucosiduronase[23], which should leave uronate residues at the reducing terminus. If this hypothesis is correct, such terminal residues must be selectively lost during the cur-



Scheme 1. Reaction scheme for the methylation analysis of heparin.

TABLE I

PROPERTIES OF IDENTIFIED CARBOHYDRATE UNITS OF HEPARIN

Cpd. No.	*Parent sugar or possible origin*[a]	*Observed derivative*			*R.T. (min)*	*Major e.i. peaks (m/z)*	*C.i. mol. wt.*		*Reference spectra*
		Parent deriv.	*Acetyl*	*Methyl*			*M − 59*	*M + 1*	
		hexitols							
1	NR. uronate		1,5	2,3,4,6	18.17	117, —	263	—	8
2	uronate		1,4,5	2,3,6	19.13	117, 233	291	—	8
3	**4** as NR. terminus		1,3,5	2,4,6	19.22	117, 129	291	—	8
4	uronate 3-sulfate or inc. O-3 methylation		1,3,4,5	2,6	19.78	117, 129	319	—	8
5	inc. O-6 methylation?		1,4,5,6	2,3	20.22	117, 129	319	—	8
11	uronate 2-sulfate		1,2,4,5	3,6	22.10	129, 233	319	—	8
		2-deoxy-(N-methylacetamido)hexitols							
6	R. hexosamine 6-sulfate		4,5,6	1,3	20.67	130 —	—	391	7
7	NR. hexosamine		1,5	3,4,6	21.06	116, 158	—	363	7
8	only 6-pos. not free OH?		5,6?	1,3,4?	21.25	130 —	—	363	—
10	hexosamine		1,4,5	3,6	21.90	116, 158	—	391	7
12	galactosamine in contam. glycosaminoglycan (DS)		1,4,5,6	3	22.25	116, 158	—	419	—
13	NR. hexosamine 6-sulfate		1,5,6	3,4	22.40	116, 158	—	391	7
14	hexosamine 6-sulfate		1,4,5,6	3	23.00	116, 158	—	419	7
17	hexosamine 3,6-disulfate (active site hexosamine)		1,3,4,5,6	—	23.60	116, 158	—	447	7
		2-acetamido-2-deoxyhexitols							
9	inc. *N*-methylation of **6**		4,5,6	1,3	21.50	129, 233	—	377	—
15	inc. *N*-methylation of **14**		1,4,5,6	3	23.15	84, 144	—	405	—
16	inc. *N*-methylation of **17**		1,3,4,5,6	—	23.25	102, 144	—	433	—

[a]NR. = nonreducing; R. = reducing.

rent scheme. However, our findings suggest that a selective chain-cleavage may well occur at hexosaminyl 6-sulfate residues.

Several sugars were also identified that had not been predicted. Compound **4**, which matched published spectra, is of interest, in that it must result either from incomplete methylation of O-3 or from a uronate 3-sulfate. Neither explanation is entirely satisfactory. Uronate 3-sulfate has not previously been reported in heparins. Compound **11**, which must represent a large fraction of iduronate 2-sulfate, gives about one-third of the peak area given by compound **4**; hence, if compound **4** represents incomplete methylation, O-3 must be particularly unreactive. Its appearance as a nonreducing terminal sugar is also of interest. Compound **12**, which has a mass spectrum identical to that of the major hexosamine derivative, but which differs in retention time, may well represent 2-amino-2-deoxy-D-galactose contributed by a small amount of contaminating dermatan sulfate.

Some evidence for incomplete methylation was found. Compound **5**, which was matched against a previously published spectrum[7], may represent incomplete methylation of O-6, or a new structural element in heparin. Compounds **9**, **15** (which is actually underneath the peak for compound **14**), and **16** did not match previously published spectra; however, their molecular weights and fragmentation patterns indicate incomplete *N*-methylation. In each case, however, these represent small fractions of the fully methylated compounds, but, interestingly, the proportion rises with the apparent degree of sulfation of the parent residue. This may serve as an indication that failure to achieve complete *N*-methylation may reflect steric factors inherent in the structure of heparin, thus providing additional structural information.

The only complete puzzle was presented by compound **8**, for which we could not find a reference spectrum. Although the structure assigned is consistent with the mass-spectral data, its presence cannot at present be explained.

The reasons for the apparent differences in the yields of hexosaminyl and glycosyluronic residues have not yet been identified, but may reflect losses due to the technique employed. Whereas the acetolysis–hydrolysis procedure used here has been reported to lead to quantitative recovery of monosaccharides when applied to oligosaccharides containing *N*-acetylhexosaminyl residues[7], it may not be so efficient with the methylated, carboxyl-reduced heparins.

Clearly, further research will be required in order to develop procedures for quantitative analysis of all of the residues in heparins, or other complex, sulfated glycosaminoglycans. The problem of loss of glycosyluronic residues will have to be solved, a more complex derivatization scheme will need to be developed, and response factors will have to be determined. Despite these requirements, however, application of the current technique may allow heparins to be "finger-printed", or compared to each other in a relative sense. Nevertheless, these preliminary results do show that such complex, sulfated glycosaminoglycans as heparins and heparan sulfates can be methylated, and that the extremely powerful, structural tool of methylation analysis is, at least, feasible with these important substances.

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