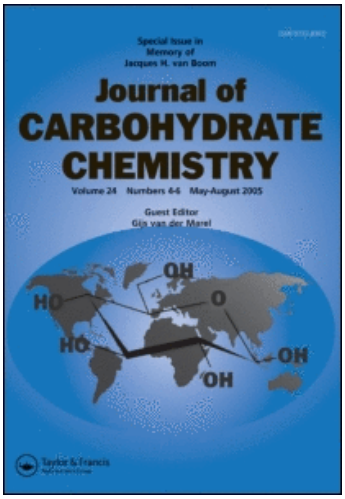

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ALKALI-INDUCED OPTICAL ROTATION CHANGES IN HEPARINS AND HEPARAN SULFATES, AND THEIR RELATION TO IDURONIC ACID-CONTAINING SEQUENCES [1]

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ABSTRACT

Under specific basic conditions, the glucosaminoglycans heparin and heparan sulfate, containing α-L-iduronic acid 2-*O*-sulfate, undergo selective epoxidation between *C*-2 and *C*-3 of this residue, with formation of a residue of 2,3-anhydro-α-L-guluronic acid. The epoxidation reaction was studied by means of ^{13}C NMR and optical rotation measurements. The optical rotation values correlate well with the composition of the reaction products as determined by ^{13}C NMR, thus indicating that the heparin- or heparan-like character of both natural and semisynthetic polysaccharides can be easily determined also through optical rotation measurements.

INTRODUCTION

Heparins and heparan sulfates are well-known biologically active natural polysaccharides belonging to the class of glycosaminoglycans (GAG). They are made up of alternating residues of an uronic acid (β-D-glucuronic **[GlcA]** or α-L-iduronic **[IdoA]**) and a hexosamine (α-D-glucosamine **[GlcN]**) joined by 1,4-glycosidic

linkages, with various *O*-sulfate, *N*-sulfate and *N*-acetyl substituents which are usually non-homogeneously distributed along the GAG chains.

Heparins and heparan sulfates originate from the same biosynthetic precursor, *N*-acetyl heparosan. However, their biosynthesis takes place in different cells for the two GAGs, and proceeds much further for heparin (with *N*-deacetylation and *N*-sulfation of glucosamine residues, conversion of **GlcA** to **IdoA** residues, and further sulfation on both residues) than for heparan sulfate.[2] Depending on the progress of their biosynthesis, heparins and heparan sulfates are composed of different proportions of the same building blocks.

The problem of defining hybrid GAGs containing both "heparin-like" and "heparan-like" sequences is especially complicated for "heparinoids" currently used in therapy or evaluated as experimental drugs, consisting of GAG mixtures or chemically-modified heparins with variable degrees of *N*- and *O*-sulfation, *N*-acetylation and content of **IdoA2SO$_3$**.

It has been suggested that a GAG should qualify as heparin only if its content in sulfamino groups largely exceeds its content in acetamido groups and the concentration of *O*-sulfate groups exceeds that of *N*-sulfate groups. It was further recommended that all the other related polysaccharides should be referred to as heparan sulfates.[3,4]

Natural heparins contain the major disaccharide unit α-L-iduronic acid 2-*O*-sulfate - α-D-glucosamine *N*-sulfate **[IdoA2SO$_3$ - GlcNSO$_3$]**, with extensive sulfation at position 6 (sequence **1** in Fig. 1) and with some of the aminosugar residues being *N*-acetylated instead of *N*-sulfated.[5] In contrast, the prevalent sequence in natural heparan sulfates (**2**) is made up of **GlcA** and *N*-acetyl-α-D-glucosamine **[GlcNAc]** residues, the content of **IdoA2SO$_3$** units being usually less than 20%.[6]

In view of the biochemical and pharmaceutical interest in this class of polysaccharides, it is important to classify rapidly a given GAG in terms of its prevalent sequences. The possibility of using the content of sulfated iduronic acid in order to define the borderline between these glucosaminoglycans prompted us to set up an easy method for the determination of **IdoA2SO$_3$**.

In previous studies on glucosaminoglycans behaviour in alkaline medium, Perlin and co-workers[7,8] and ourselves[9-11] showed that **IdoA2SO$_3$** residues undergo regio- and stereospecific epoxidation (sequence **3**) without substantial depolymerization. The following hydrolysis of the epoxide selectively gives rise to α-L-galacturonic acid **[GalA]** (sequence **4**) or to nonsulfated **IdoA** residues (sequence **5**) depending on the experimental conditions (Figure 1).

In this work, this reaction was monitored through optical rotation and ^{13}C NMR measurements.

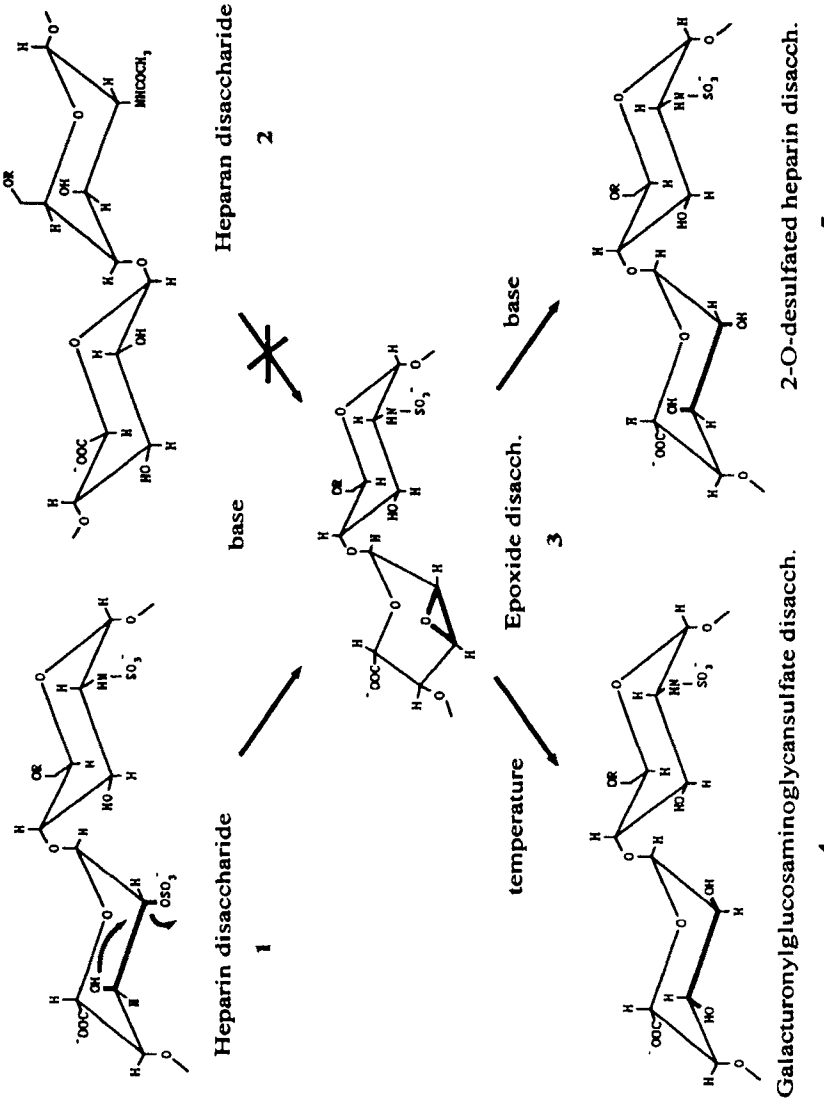


FIG. 1. Heparan sulfate and heparin reactions in alkaline medium (only major disaccharide units shown; R = H or SO_3^-).

RESULTS AND DISCUSSION

Alkali treatment of glucosaminoglycans containing **IdoA2SO$_3$** afforded 2,3-epoxigulonic acid units. The formation of 2,3-epoxigulonic acid residue is a base-induced intramolecular displacement by *O*-3 of the axial 2-sulfate group on the **IdoA2SO$_3$** unit.[7,9]

The oxirane ring may undergo selective hydrolysis either to **GalA** (sequence **4**), under neutral or weakly basic conditions at high temperature, or to desulfated **IdoA** (sequence **5**), under stronger basic conditions and at lower temperatures.[8,10]

The various species were determined by ^{13}C NMR spectroscopy (assignments by Jaseja and co-workers[7]). The spectra of a pig mucosal heparin and its major products of reaction with base are reported in Figure 2.

The variation of optical rotation during the reactions in alkaline medium has been monitored: the epoxide formation induces a sharp increase, whereas the subsequent hydrolysis of the epoxide induces a decrease of optical rotation.

In order to study the influence of different structural modifications, heparin derivatives prepared using well-known reactions, such as solvolytic *N*-desulfation,[12] and *N*-acetylation,[13] were studied. These chemical modifications on hexosamine residues did not produce any significant variation of specific optical rotation of heparin epoxides. In fact, the ^{13}C NMR spectra (not shown) indicated that the oxirane rings did not open under conditions of *N*-desulfation, either by acid hydrolysis or by DMSO; this permitted the preparation of a large number of heparin derivatives for a systematic study of the functional role of sulfate groups.[14] Physico-chemical data for some heparin derivatives are reported in Table 1.

Since the data indicated a dependence of variation of optical rotation only on epoxidation and subsequent hydrolysis of the epoxide ring, the kinetics of these reactions was studied (under controlled conditions) in order to evaluate the feasibility of determining the percentage of **IdoA2SO$_3$** in different GAGs simply through optical rotation measurements.

The kinetics of the reactions on beef lung heparin was similar to first order consecutive reactions; the mathematical expression is reported in Table 2.

The rate constant (k_1) of the first reaction (epoxidation) was determined from the decreasing area of the *C*-1 peak of **GlcNSO$_3$** (100.3 ppm) in **IdoA2SO$_3$ - GlcNSO$_3$** sequences and subsequent extrapolation to zero time: the rate constant was $k_1 = 0.95$ h^{-1} (Figure 3).

The calculated and experimental values of molar fraction of **IdoA2SO$_3$** differ slightly (Figure 4). This difference may be explained by the existence of two stable

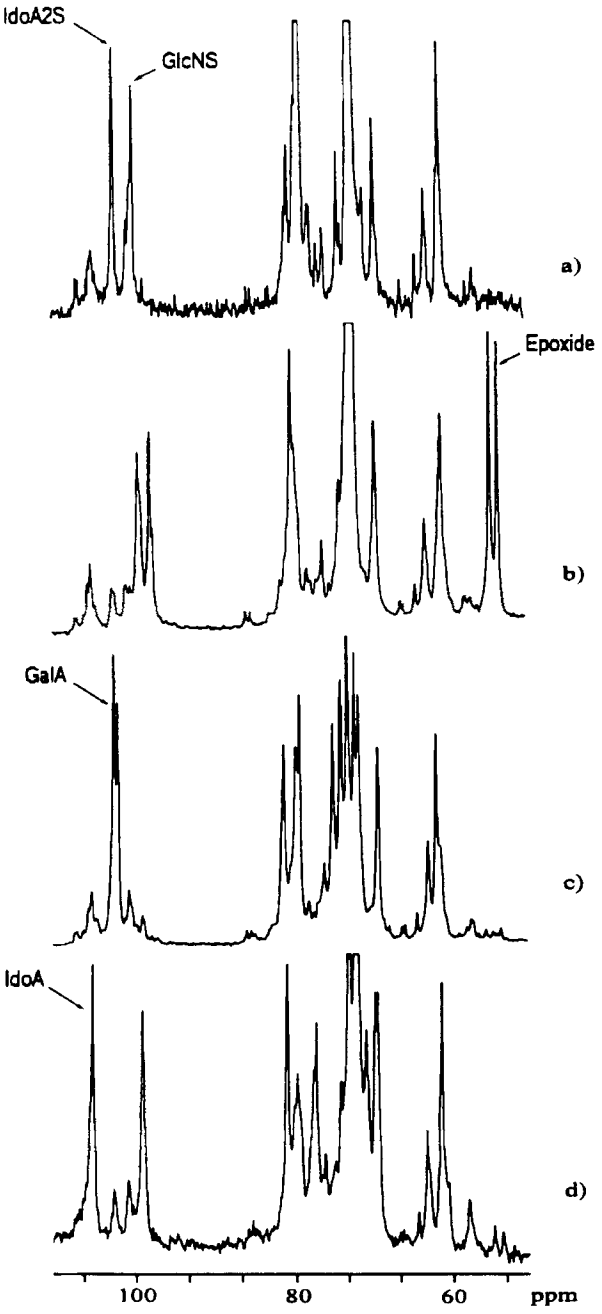
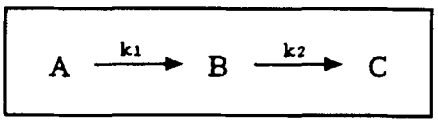


FIG. 2. ^{13}C NMR spectra of: a) pig mucosal heparin; b) epoxide derivative; c) galacturonyl derivative; d) 2-O-desulfated heparin.

TABLE 1. Physico-chemical data of heparin and heparin derivatives: pig mucosal heparin **[H]**; *N*-desulfated heparin **[N-des H]**; *N*-acetylated heparin **[N-Ac H]**; epoxide **[Epox]**; *N*-desulfated epoxide **[N-des Epox]**; galacturonyl **[GalGGS]** and *N*-desulfated galacturonyl **[N-des GalGGS]** derivatives.

Compound	R (SO_3^-/COO^-)	S%	P.M. (average)	NSO_3^- (mol. fr.)	$[\alpha]^{25}$ (at 546nm)
H	2.1	11.6	12,000	0.94	+59°
N-des H	1.2	8.9	10,560	0.05	+68°
N-Ac H	1.3	8.5	10,800	0.06	+65°
Epox	1.4	9.2	10,800	0.94	+107°
N-des Epox	0.5	6.7	9,400	0.03	+108°
GalGGS	1.3	8.8	10,680	0.90	+18°
N-des GalGGS	0.4	6.5	9,300	0.07	+28°

TABLE 2. Mathematical expressions of first-order consecutive reactions.



$A/Ao = \alpha$

$B/Ao = \beta$

$C/Ao = \gamma$

$k_1 t = \tau$

$k_2/k_1 = K$

$$\alpha = e^{-\tau}$$

$$\beta = (1/K-1)(e^{-\tau}-e^{-K\tau})$$

$$\gamma = 1 + (1/1-K)(Ke^{-\tau}-e^{-K\tau})$$

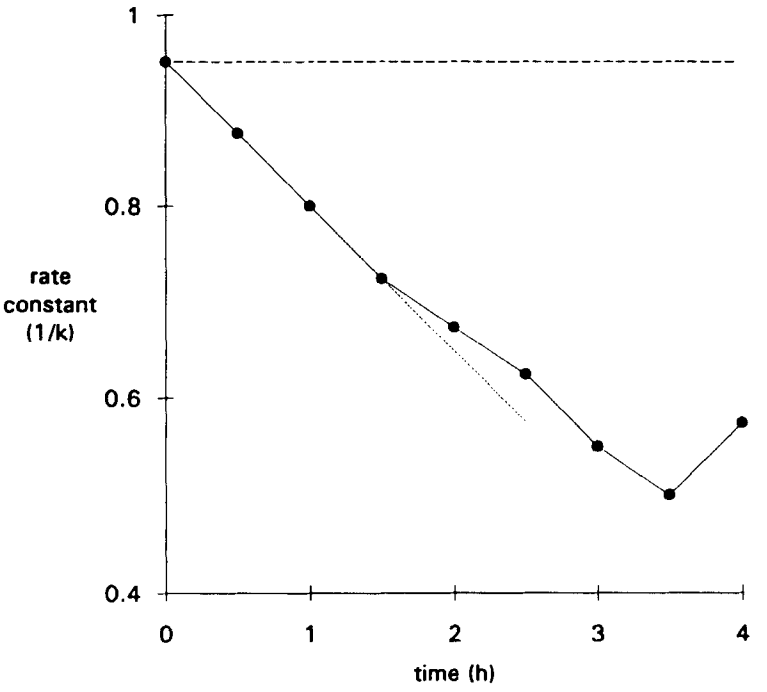


FIG. 3. Experimental rate constant *vs.* reaction time.

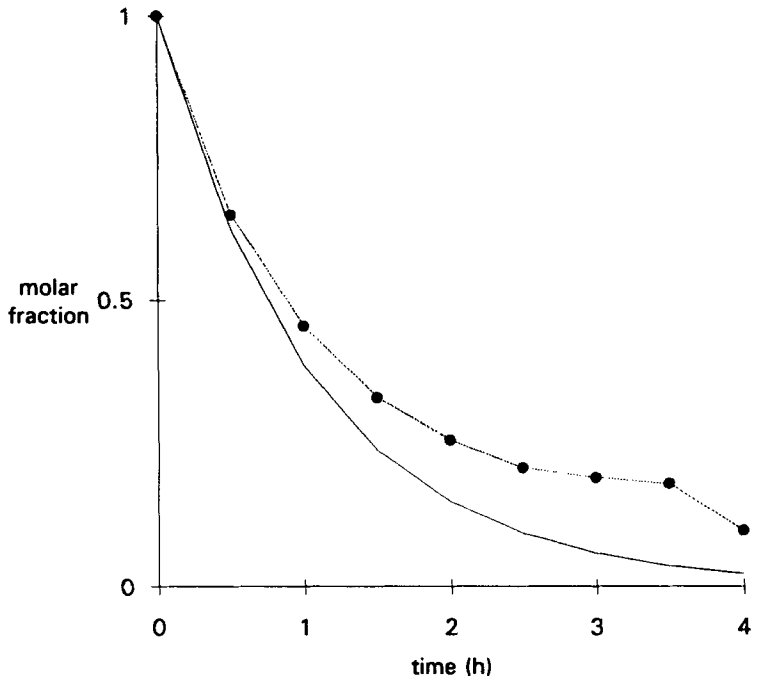


FIG. 4. Molar fraction of **IdoA2SO$_3$** *vs.* reaction time [(....) experimental data, by NMR; (—) calculated].

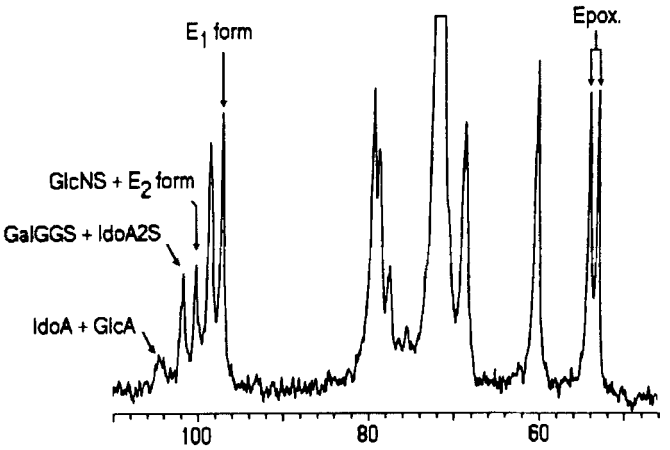


FIG. 5. ^{13}C NMR spectra of species obtained after 90 minutes reaction (60 °C - 1N NaOH) (the arrows indicate the considered peaks).

epoxide conformers (^5H_O and $^OH_5$ conformations), suggested by Rej and Perlin,[8] with anomeric uronic carbon resonances respectively at 97.3 ppm (**E_1 form**) and 100.3 ppm (**E_2 form**). The two different half-chair conformations of epoxide species could account for the subsequent hydrolysis to either galacturonic or iduronic acids according to the Fürst-Plattner rule.[15] Alternatively, the minor species (**E_2 form**) might reflect only sequence effects, *e.g.* due to *N*-acetylated glucosamine residues.

The percentages of the species involved in the reactions, determined on the basis of resonances shown in Figure 5, are in Table 3.

The molar fractions of two epoxide species (E_1 and E_2) and their addition are compared in Figure 6 with the calculated kinetic values; the epoxide in E_2 form represents less than the 10% of overall 2,3-epoxigulonic species.

The quantitative determination of all species involved in the reactions permitted to calculate the rate constant for the subsequent hydrolysis: $k_2 = 0.038$ h^{-1}. Comparison of k_1 and k_2 values indicates that the epoxide ring opening is 25 times slower than epoxidation, and this made possible the isolation of the epoxide. Mathematical expression of consecutive reactions kinetics allowed us to calculate the highest obtainable molar fraction of epoxide species in this reaction mixture, and the reaction time at which the maximum yield is obtained. The experimental and calculated data are in good agreement (Table 4).

Considering the optical rotation value of all species involved in the reactions, it is possible to plot the theoretical value of optical rotation *vs.* time; a good agreement

TABLE 3. Percentages of uronic acid residues at different times during the reaction on beef lung heparin with alkali, as determined by ^{13}C NMR.

time (h)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Iduronic acid 2-O-sulfate	85.0	52.2	32.3	19.7	12.3	8.2	4.4	2.8	1.4
Epoxide [E_1 form]	-	31.3	45.2	54.9	65.8	67.1	65.2	69.9	72.2
Epoxide [E_2 form]	-	1.5	3.2	4.2	4.8	4.8	5.9	6.3	3.5
Desulfated uronic acids (IdoA + GlcA)	15.0	11.9	14.2	16.9	11.0	15.1	16.3	14.0	15.3
Galacturonic acid	-	3.0	5.2	4.2	6.2	4.8	8.1	7.0	7.6

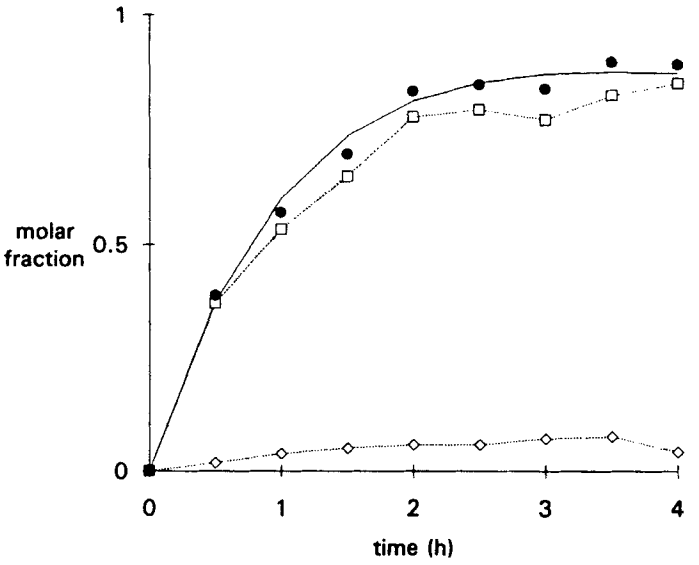


FIG. 6. Molar fractions of epoxide species [(—) calculated; E_1 (□) and E_2 (◇) form experimental data by ^{13}C NMR; (●) their addition].

TABLE 4. Calculated data in comparison with experimental ones for kinetic studies under standard conditions.

$k_1 = 0.95\ h^{-1}$	$k_2 = 0.038\ h^{-1}$
$K = k_2/k_1 = 0.04$	$1/K = 25$

	calculated	experimental
$[B/Ao]_{max} = K^{K/1-K}$	0.874	0.896
t_{max} (h)= $[1/k_1(K-1)]$ lnK	3.53	3.5

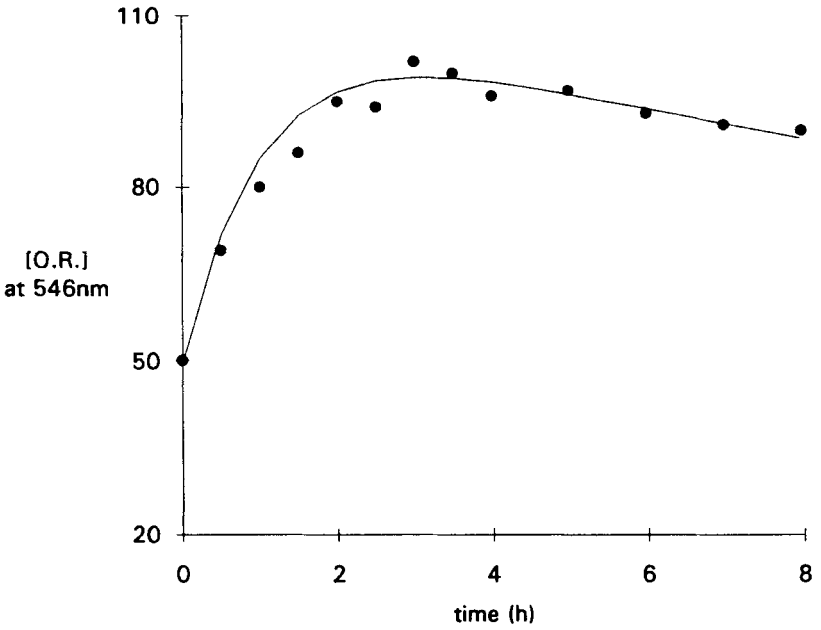


FIG. 7. Optical rotation values *vs.* time in alkaline medium reaction [(—) calculated; (•) experimental].

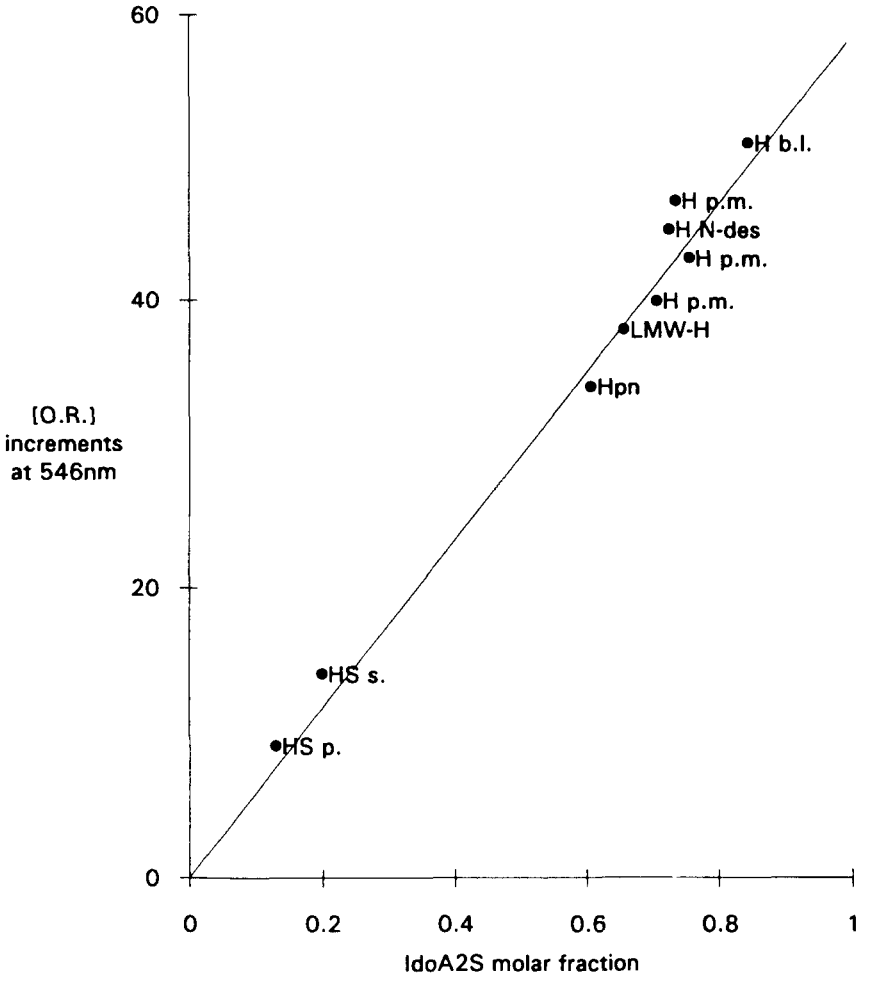


FIG. 8. Increments of optical rotation values after reaction under standard conditions *vs.* initial **IdoA2SO$_3$** percentages (as determined by NMR).

with experimental data is illustrated in Figure 7. The same figure also accounts for the experimental observation that the value of the optical rotation increases owing to the positive contribution of epoxide residues and then decreases with the hydrolysis of the epoxide ring.

However, the difference between the optical rotation value after epoxidation and the starting value depends on the initial percentage of **IdoA2SO$_3$** residues.

As shown in Figure 8, the increment of optical rotation in the epoxidation step, upon standard alkaline treatment of glucosaminoglycans (a number of heparins, an

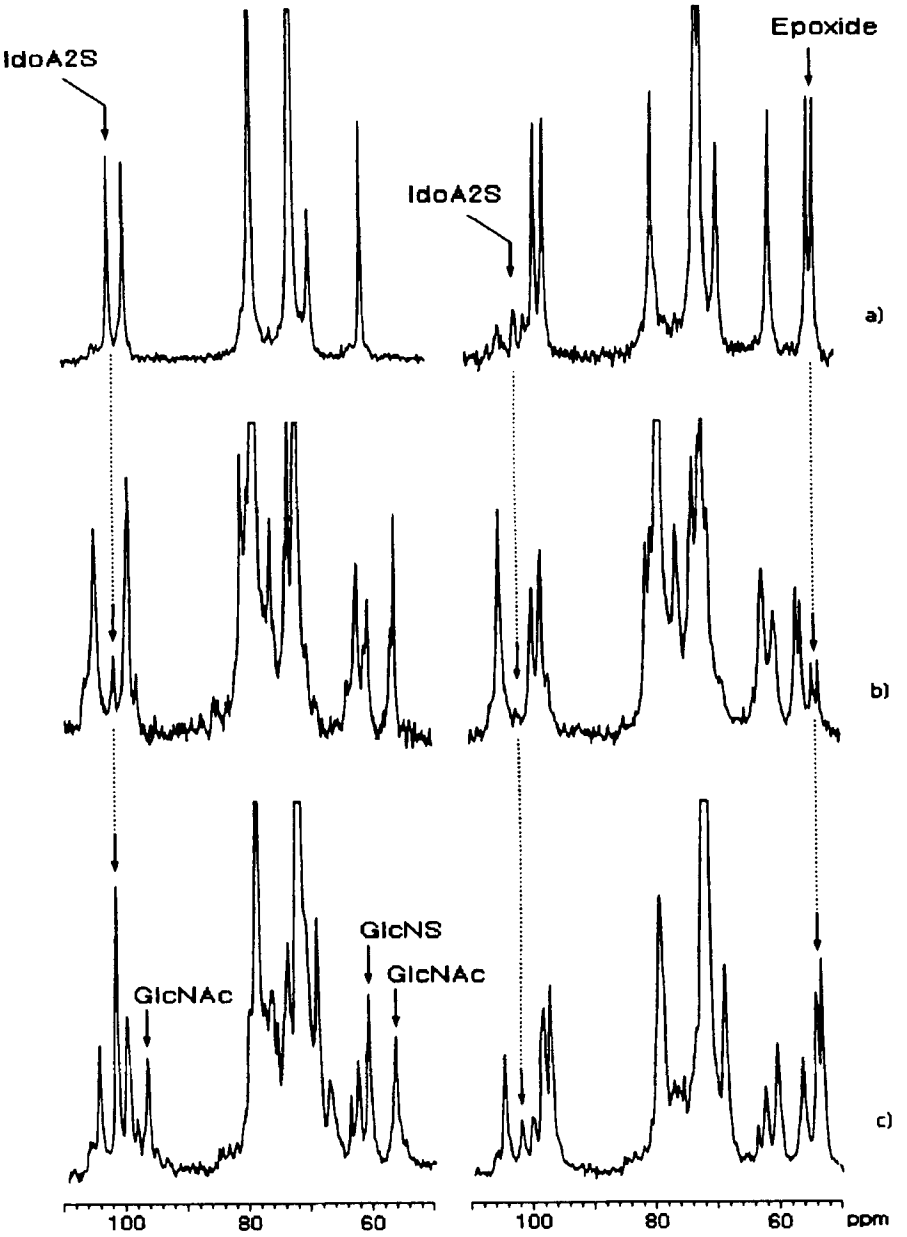


FIG. 9. ^{13}C NMR spectra of a) beef lung heparin, b) spleen heparan sulfate, c) commercial heparinoid extract (left) and their epoxide derivatives (right) obtained under standard reaction conditions.

N-desulfated heparin, two heparan sulfates, and an heparinoid), is a linear function of the initial molar fraction of **IdoA2SO$_3$**, as determined by ^{13}C NMR. The regression line is:

Iduronic acid 2-*O*-sulfate % = Optical rotation increment x 1.72

The ^{13}C NMR spectra of beef lung heparin, spleen heparan sulfate, heparinoid extract and the corresponding epoxide derivatives are reported in Figure 9. In the spectra of the heparinoid, the peaks at 96.7 and 56.4 ppm of *C*-1 and *C*-2 of **GlcNAc** in **IdoA2SO$_3$ - GlcNAc** sequences "qualify" it as a partially *N*-desulfated, *N*-acetylated heparin.

CONCLUSIONS

A simple optical rotation measurement after an alkali treatment under standard conditions up to the epoxidation step permits determination of the percentage of **IdoA2SO$_3$** residues in glucosaminoglycans. In this way it is possible to differentiate natural and semisynthetic heparins and heparan-like glucosaminoglycans on the basis of their different contents in these residues.

This method is a useful, alternative tool to NMR analysis for defining the heparinic or heparanic character of commercial glycosaminoglycan drugs and because of its quantitative nature and simplicity it is an improvement on other techniques requiring enzymatic (heparinase) or chemical (HNO_2) degradation.

EXPERIMENTAL

Materials. - Beef pancreas **[HS p.]** and spleen **[HS s.]** heparan sulfates, LMW heparin **[LMW-H]** and pig mucosal heparins **[H p.m.]** were from Opocrin (Modena, Italy). Beef lung heparin **[H b.l.]** was from Biofer (Modena, Italy). The heparinoid **[Hpn]** was extracted from commercial Hemovasal® tablets (Manetti-Roberts) following a general GAG extraction procedure,[16] after filtration of water-insoluble components.

General methods. - Optical rotations were recorded at 25 °C with an Optical Activity Ltd. polarimeter, at 1% concentration in H_2O. The ^{13}C NMR spectra were recorded with a Varian Gemini 200 spectrometer at room temperature, at 50.3 MHz in D_2O solutions. Chemical shifts are given in ppm with reference to the internal standard 2,2,3,3-d_4-3-trimethylsilylpropionic acid, sodium salt. The sulfate/carboxyl molar ratios and sulfur percentages were obtained by conventional potentiometric titration, with a Mettler DL 25 titrator. The average molecular weights were determined with HPLC-GPC analysis with Waters Model 510 HPLC instrument, using a Waters Model R 401 refraction index detector.

Epoxidation reaction. - The standard reactions as well as the kinetic studies were performed in 4% GAG aqueous solutions at 60 °C and 1N NaOH. Standard reactions were carried out for 210 minutes. The GAGs were recovered, after neutralization and dialysis (cut off 1.5 kD), by lyophilization.

Hydrolysis reaction. - The epoxide rings were hydrolyzed either by heating (leading to **GalA** derivatives) or with base (leading to nonsulfated **IdoA** derivatives). Reactions and isolation of products were performed essentially as described by Jaseja et al.[7]

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