Acid-Base and Chiroptical Properties of N-Desulfated Heparin

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ABSTRACT: Acid-base properties of N-desulfated heparin with almost no residual N-sulfate groups (NDHEP), as shown by ^{13}C NMR, are investigated by monitoring the reaction of sodium hydroxide with N-desulfated heparinic acid (NDHEPH) by potentiometric, conductometric, viscometric, and circular dichroism measurements. It is shown that positive charges of glucosammonium residues generated by N-desulfation are stoichiometrically balanced by part of the OSO_3^- present in NDHEPH, and in NDHEPNa as well. Glucosammonium residues are converted to glucosamine ones in alkaline medium (pH >8) only. The apparent acid strength of glucosammonium residues ($pK^a_{1/2} \sim 9.8$) in NDHEP is higher than in glucosamine-containing low molecular weight or polymeric compounds. Optical activity of amino chromophores in NDHEP and of sulfamate chromophores in heparin is discussed on the basis of comparison with the chiroptical behaviors of model compounds. Contributions to the 210-nm CD band observed for heparin and NDHEP are evaluated. Structure, acid-base reactions, and chiroptical properties of NDHEP being better understood, it is shown that CD differences do exist between sodium and calcium salts though no residual $NHSO_3^-$ groups are present. Correlation of these differences to calcium intramolecular affinity is discussed.

Introduction

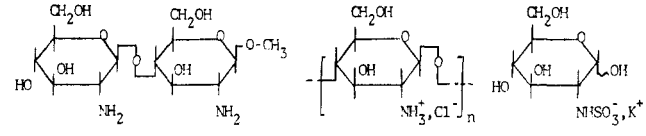
The understanding of the chemical structure of heparin has been considerably improved since this mucopolysaccharide was discovered.[1] Heparin is now known to be a mixture of linear macromolecules of different molecular weight and to consist of alternating hexuronic acid residues and glucosamine-based residues (1→4)-glycosidically linked. The former are largely α-L-idopyranosyluronic acid 2-sulfate; however, nonsulfated α-L-idopyranosyluronic acid and β-D-glucopyranosyluronic acid are also present. The latter are largely 2-deoxy-2-sulfamido-α-D-glucopyranose 6-sulfate which are sometimes replaced by 2-acetamido-2-deoxy-α-D-glucopyranose.[2] Heparin is actually more complex as the proportion of the various residues differs from one macromolecule to another so that commercial compounds can be separated into many fractions slightly different insofar as molecular weight, structure, and physicochemical and biological properties are concerned.[3] Heparin is extensively studied for its biological properties, and efforts are made to correlate the various structural aspects to its anticoagulant and antilipemic activities.[4] Heparin is also a worthwhile optically active polymer that deserves special attention for three main reasons: (1) its polyanionic structure is based on rather rigid heterocyclic units linked together through ether bonds, (2) various chromophores are present, some being ionizable, and (3) it may form complexes with many cations, especially alkaline-earth ones.[5]

In previous papers,[5,6] we have reported investigations of the chiroptical properties of heparinic acid derived from a defined sample of commercial heparin. The effects of various salts (alkali-metal and alkaline-earth chlorides) on the 210-nm CD band of heparin were considered for different values of the degree of neutralization of sulfate and carboxylate groups and for different counterions in aqueous solutions. Variations of ellipticity were related to the acid-base properties of uronic carboxyl chromophores, thus showing that ellipticity at 210 nm can be used to monitor the counterion exchange at uronic sites of a given heparin salt in the presence of mineral salts with different cations. In particular, it has been shown that sodium heparinate turns to calcium heparinate in the presence of small amounts of Ca^{2+} ions while large amounts of Na^+ ions are required to turn calcium heparinate to sodium heparinate. This difference has been considered as reflecting the higher affinity of heparin for calcium ions over sodium ions.[6]

Whether the affinity of heparin for divalent cations depends on classical interactions with ionic groups governed by charge density or on the formation of a well-defined complex involving neighboring $NHSO_3^-$ is still questioned. Recently, CD and NMR spectra of sodium and calcium salts of heparin and of corresponding N-desulfated heparin have been reported that suggest, among other possibilities, that carboxylic groups strongly bind Ca^{2+} cations and that sulfamido groups of an adjacent sugar residue simultaneously engage in a weaker electrostatic interaction so as to stabilize the complex. This proposition has been based on the fact that no difference was detected between the CD spectra, and NMR spectra as well, of Na^+ and Ca^{2+} salts of N-desulfated and N-acetylated N-desulfated heparins.[7]

Because N-desulfation actually yields a complex polyelectrolytic system where $NHSO_3^-$ negative charges are replaced by NH_3^+ positive ones and because preliminary investigations had shown that CD spectra of Na^+ and Ca^{2+} salts of N-desulfated heparin did exhibit differences, we decided to investigate in more detail the ionization behavior of N-desulfated heparin and the cation dependence of its optical activity as monitored by CD.

In this paper, we report the results obtained for a well-defined sample of N-desulfated heparin. The absence of residual $NHSO_3^-$ groups is first checked by ^{13}C NMR. Then acid-base reactions in salt-free solutions are monitored by combining potentiometric, conductometric, viscometric, and circular dichroism measurements. In order to better understand the ionic behavior and chiroptical properties of N-desulfated heparin, comparison is made with the optical activity of the following model compounds: methyl diamino-β-chitobiose (I), chitosan (II) and 2-deoxy-2-sulfamido-D-glucose (III).



I II III

Finally, CD spectra of sodium and calcium salts of N-desulfated heparin are reexamined in regard to cation exchanges.

Experimental Section

Materials. N-Desulfated heparin was prepared by solvolytic N-desulfation of Choay heparin (lot H108 dialyzed against distilled

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water using Spectra-Por 3 tubing) according to the Inoue–Nagasawa procedure.[8] An aqueous solution of 800 mg of this sample was percolated through a cation-exchange column loaded with sodium sulfonate resin, further adjusted to pH 7.5 by addition of sodium hydroxide as recommended,[9] dialyzed against pure water, and finally freeze-dried to yield pure N-desulfated heparin sodium salt (NDHEPNa). Methyl diamino-β-chitobiose was prepared by Delmotte and Gatellier.[10] Chitosan was purchased from Sigma (lot 91F-0401). The sample was acidified by HCl, dialyzed against distilled water, and freeze-dried. Sodium salts of chondroitin 4-sulfate (Sigma, lot 88C-0130) and dermatan sulfate (Sigma, lot 109C-3910) were conditioned by using the procedure described above for NDHEPNa. 2-Deoxy-2-sulfamido-D-glucose was obtained as such from Choay Institute. Mineral salts and chemical reagents were analytical grade.

^{13}C NMR. $^{13}C\{^1H\}$ NMR spectra were recorded at 20.11 MHz for solutions in D_2O at room temperature using a Bruker WP 80 spectrometer operated by the Service Commun "Recherche" of the University of Rouen.

Potentiometry. Potentiometric titrations were carried out at 25.0 °C with a Radiometer pH M-64 pH meter, fitted with an Ingold combined glass electrode standardized at pH 4.005 (50 mM potassium hydrogen phthalate buffer) and at pH 9.196 (50 mM borax buffer). pH values were measured on solutions maintained under a nitrogen atmosphere after stirring had been stopped.

Viscometry. Viscometric measurements were performed at 25.0 °C with a Sepema "Viscomatic" viscometer.

Conductometry. Conductometric titrations were carried out according to Casu and Gennaro[11] using a Radiometer CDM3 conductometer fitted with a Tacussel cell.

Circular Dichroism. CD spectra were recorded with a Jasco J40B dichrometer at room temperature (~21 °C). The double monochromator and cell compartment were purged with nitrogen. Ellipticities at 200 and 210 nm were determined by setting the monochromator at the selected wavelength. For circular dichroism titrations, a stop-flow cell (l = 0.03 dm) was used. Because mean residue molecular weights of heparinic compounds are not precisely known, CD data have been expressed in terms of measured ellipticity (Θ) instead of molar ellipticity [Θ]. Cell path length and concentration are given in captions.

Infrared Measurements. IR spectra were recorded with a Perkin-Elmer 177 spectrometer. Films were made on Irtran 2 plates by evaporation of solutions of NDHEPNa adjusted to different pH values by addition of suitable amounts of 1 M NaOH or HCl. Deuterated water was present in solutions derived from those used for ^{13}C NMR investigations.

Results and Discussion

1. ^{13}C NMR of N-Desulfated Heparin. N-Desulfation was carried out by hydrolysis of the pyridinium salt of a sample of commercial heparin (HEPNa) according to the procedure described by Inoue and Nagasawa.[8] The resulting N-desulfated heparin was carefully turned into its sodium salt form (NDHEPNa). Figure 1 shows ^{13}C NMR spectra of NDHEPNa and of the parent HEPNa. The latter exhibits a peak at 22.6 ppm corresponding to N-acetylated glucosamine residues and, thus, belongs to A-type heparins according to Perlin.[12] The other peaks have been correlated to structural characteristics (Table I) on the basis of assignments reported in the literature.[13,14]

N-Desulfation caused many changes in the ^{13}C NMR spectra of HEPNa. Heights of peaks 6 and 3, located at 97.5 and 58.4 ppm in the HEPNa spectrum and assigned respectively to C1 and C2 of N-sulfated residues, are very much decreased in the NDHEPNa spectrum. It is of interest to point out that peak 3 vanishes almost completely while a small peak (peak 13) remains observable at 97.8 ppm. The latter might be considered as evidence for the presence of residual N-sulfate groups. However, the fact that peak 3 disappeared almost completely argues in favor of a high degree of desulfation. As for peak 13, it is regarded as a small peak overlapped by peak 6 in the native HEPNa spectrum. No assignment is feasible at the moment. However, it is concluded that N-desulfation is practically total in our sample of NDHEP. Amid the appearing peaks (peaks 9, 10, and 11) located at 54.7, 63.3, and 91.5 ppm, respectively, peaks 9 and 11 can be reasonably assigned to the C2 and C1 atoms of the N-desulfated glucosamine residues which are protonated under the experimental conditions as shown below. Accordingly, these two peaks correspond to peaks 6 and 3 of heparin shifted because of N-desulfation. On the basis of similar shifting, peak 10 could be assigned to the C5 or C3 atoms

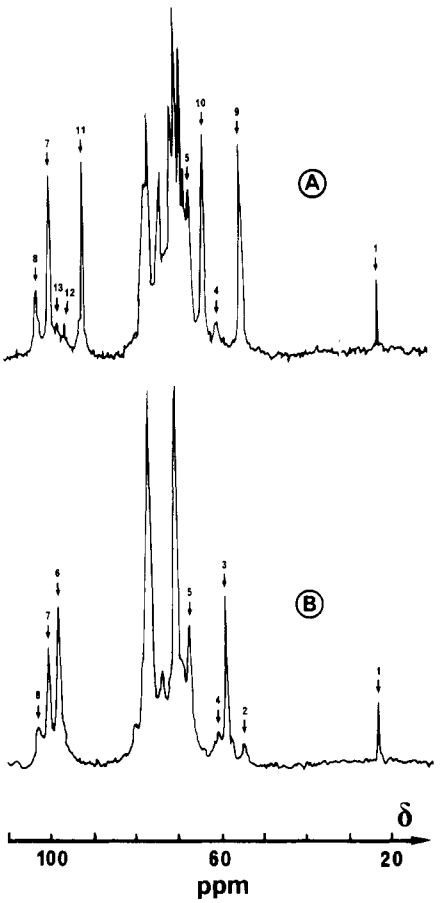

Figure 1. ^{13}C NMR spectra of NDHEPNa (A) and parent HEPNa (B). Assignments of peaks marked by arrows are listed in Table I.

Table I
Assignments of Peaks of ^{13}C NMR Spectra of HEPNa and NDHEPNa in Deuterated Water at Room Temperature[a]

code[b]	HEPNa	NDHEPNa	assignment[c]
1	22.6	22.6	C (NAc)
2	54.4		C2 (GlcNAc)
3	58.4		C2 ($GlcNSO_3^-$)
4	60.8	60.8	C6 (GlcN-6-OH)
5	66.8	66.8	C6 (GlcN-6-OSO_3^-)
6	97.5		C1 ($GlcNSO_3^-$)
7	100.0	100.0	C1 ($IdUASO_3^-$)
8	102.6	102.6	C1 (GlcUA)
9		57.4	C2 ($GlcNH_3^+$)
10		63.3	?
11		91.5	C1 ($GlcNH_3^+$)
12		95.5	C1 (GlcNAc)
13		97.8	?

[a] Chemical shifts in ppm, peak 1 being taken as internal reference. [b] Peak numbers in Figure 1. [c] Abbreviations used: $IdUASO_3^-$, α-L-idopyranosyluronic acid 2-sulfate; GlcUA, β-D-glucopyranosyluronic acid; GlcNAc, 2-acetamido-2-deoxy-α-D-glucopyranose, $GlcNSO_3^-$, 2-deoxy-2-sulfamino-α-D-glucopyranose; GlcN, α-D-glucopyranose with unspecified 2-substituent; $GlcNH_3^+$, ammonium form of 2-amino-2-deoxy-α-D-glucopyranose.

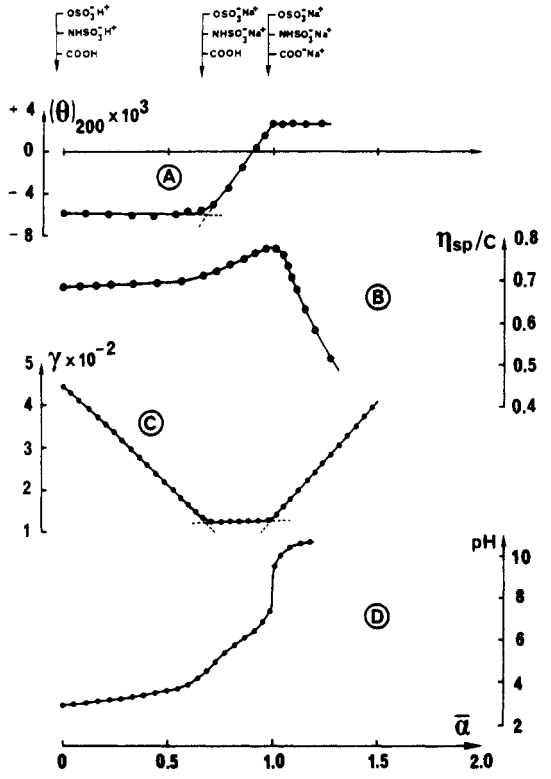

Figure 2. Variations of ellipticity (A; (Θ) at 200 nm in degree, l = 0.03 dm), viscosity (B; η_{sp}/C in 100 cm^3/g), conductivity (C; γ in μS), and pH (D) with the degree of neutralization, $\bar{\alpha}$, of a salt-free solution of HEPH (C = 0.075 g/100 cm^3) at 25.0 °C.

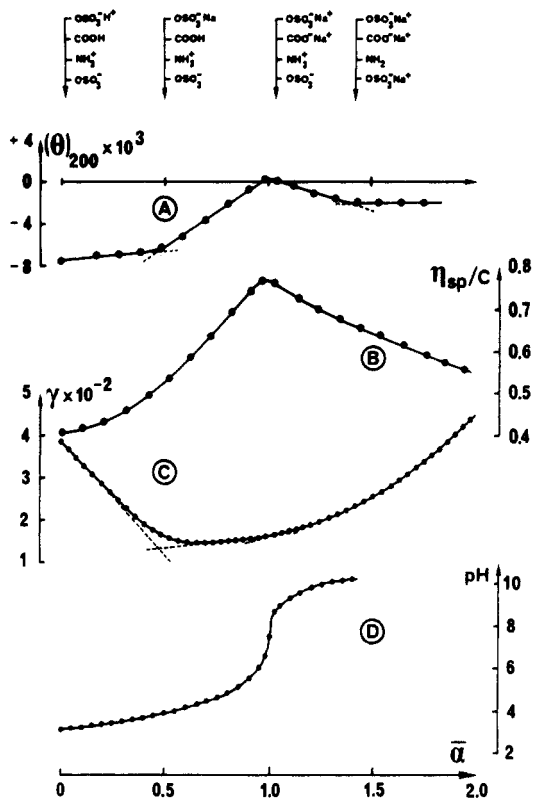

Figure 3. Variations of ellipticity (A; (Θ) at 200 nm in degree, l = 0.03 dm), viscosity (B; η_{sp}/C in 100 cm^3/g), conductivity (C; γ in μS), and pH (D) with the degree of neutralization, $\bar{\alpha}$, of a salt-free solution of NDHEPH (C = 0.072 g/100 cm^3) at 25.0 °C.

of the N-desulfated glucosamine residues whose signals are originally at ca. 70 ppm in HEPNa.[14] Many other perturbations occur in the 70–80 ppm range that cannot be simply resolved.

2. Acid–Base Reactions of HEPH and NDHEPH. Heparinic acid (HEPH) and N-desulfated heparinic acid (NDHEPH) were obtained by percolation through cation-exchange columns (H^+ form) of salt-free HEPNa and NDHEPNa aqueous solutions, respectively. Acid–base reactions caused by the addition of sodium hydroxide to HEPH and NDHEPH solutions ($C \sim 0.075$ g/100 cm^3) were monitored by potentiometric, viscometric, conductometric, and circular dichroism measurements at 200 nm. Both the presence of several functional groups able to generate electric charges on polymer chains and the presence of positive and negative charges in the case of NDHEPH cause complications in quantifying the various chemical states of the two types of macromolecules. Arbitrarily, the degree of neutralization was given the value $\bar{\alpha} = 1.00$ for chemical states corresponding to the wave of potential observed close to neutral pH for both compounds.

Figure 2 shows circular dichroism, viscometric, conductometric, and pH titration curves of HEPH. As already discussed,[5] OSO_3^-, H^+ and $NHSO_3^-$, H^+ acid groups are neutralized first in the $0 < \bar{\alpha} < 0.69$ range, where pH and viscosity increase slightly while ellipticity remains almost constant, in agreement with the fact that sulfate and sulfamate are ionized whatever the counterion. In this range of $\bar{\alpha}$ values, conductivity decreases linearly as H^+ counterions are replaced by Na^+ counterions.[11]

In the range $0.69 < \bar{\alpha} < 1.00$, carboxyl groups ionize to carboxylate ones. Waves of potential observed by measurement of pH correspond to breaks in the other titration curves. These breaks are sharp on CD and conductometric curves, but only the end of the neutralization of COOH groups ($\bar{\alpha} = 1.00$) is clearly detectable by viscometry. For $\bar{\alpha} > 1.00$, CD remains constant, thus showing that no more chemical changes are occurring and that conformational changes reflected by decreasing viscosity do not affect chiroptical properties.[5]

Figure 3 shows data obtained for NDHEPH. $\bar{\alpha} = 1.00$ is well marked in CD and viscometric curves. Other critical $\bar{\alpha}$ values are observed at $\bar{\alpha}_1 = 0.47$ in CD and conductometric curves and at $\bar{\alpha}_2 \sim 1.38$ in the CD titration curve. Neither conductometric nor viscometric curves clearly reflect the latter.

In the following, attempts are made to correlate these particular $\bar{\alpha}$ values to acid–base reactions involving the various ionic and ionogenic groups of NDHEP where $NHSO_3^-$ groups are no longer present and are replaced by NH_3^+ neutralized intramolecularly by part of the OSO_3^- groups.[15] Upon addition of sodium hydroxide, the various groups must react according to their acid strength.

Compared with data obtained for heparin, the first $\bar{\alpha}$ range ($0 < \bar{\alpha} < 0.47$) can be assigned to the neutralization of free OSO_3^-,H^+ groups based on the following features: pH is low and increases slightly with $\bar{\alpha}$, conductivity decreases almost linearly as for HEP, viscosity increases slightly, in agreement with the absence of major change in charge density, and ellipticity remains almost constant.

Above $\bar{\alpha} = 0.47$, COOH groups ionize up to $\bar{\alpha} = 1.00$, in agreement with the following features: small change of conductivity, sharp increase in viscosity due to increasing charge density, and linear variation of ellipticity resembling that observed for HEP during the ionization of COOH groups. It is noteworthy that the apparent $pK^a_{1/2}$ of COOH groups is much lower for NDHEP (ca. 4.6, pH value at $\bar{\alpha} = 0.735$) than for HEP (ca. 5.7, pH value at $\bar{\alpha} = 0.845$) under similar experimental conditions. It is known that charge density strongly affects the ionization behavior of weak acid groups in polyelectrolytes.[16] In the present cases, charge density is much smaller in NDHEP than in HEP especially because NH_3^+ charges intramolecularly neu-

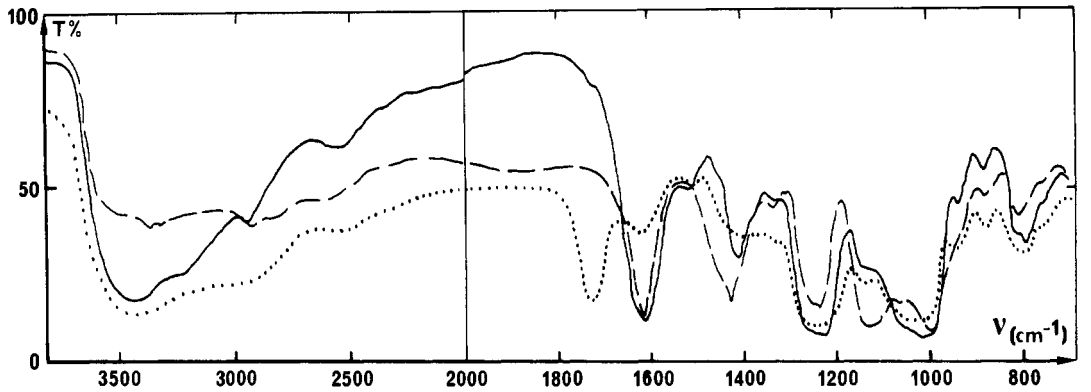

Figure 4. Infrared spectra of films cast on Irtran 2 plates from NDHEP solutions at pH ~2 (···), pH ~7 (—), and pH ~11 (---).

tralize part of the OSO_3^- anionic groups. Accordingly, neutralizations of OSO_3^-,H^+ and COOH are much less differentiated than in HEP. This point might well account for the fact that NDHEPH titration curves do not exhibit sharp breaks as HEPH ones do. The fact that neutralization occurs intramolecularly is supported by the absence of turbidity.

Above $\bar{\alpha} = 1.00$, the potentiometric titration curve of NDHEP looks like that of HEP; however, pH values in the range $1.00 < \bar{\alpha} < 1.38$ are ca. 0.8 pH unit below those found for HEP. Other particulars need to be pointed out: (1) the conductimetric curve is bent, and conductivity does not increase as fast as for HEP; (2) viscosity does not decrease as fast as for HEP; (3) ellipticity varies in the range $1.00 < \bar{\alpha} < 1.38$ while it remains constant for HEP. It is for $\bar{\alpha} > 1.38$ only that ellipticity becomes constant, i.e., beyond the $\bar{\alpha}$ value corresponding to smooth slope changes observed in viscometric and conductometric titration curves.

Accordingly, NDHEP is a polyampholyte at neutral pH, and glucosammonium positive charges are balanced by part of the OSO_3^- negative charges. The zwitterionic form thus obtained neutralizes in the $1.00 < \bar{\alpha} < 1.38$ range, turning OSO_3^-,NH_3^+ to OSO_3^-,Na^+ and NH_2 as presented in Figure 3.

This interpretation is supported by infrared spectra of NDHEP (Figure 4). In acid medium, NDHEP shows a band at 1715 cm^{-1} corresponding to $\nu_{C=O}$ in COOH groups and a broad band in the 2500–3000-cm^{-1} range resulting from the overlapping of ν_{COOH}, ν_{CH}, ν_{CH_2}, and $\nu_{NH_3^+}$.[17] In contrast, at neutral pH, $\nu_{C=O}$ is found at 1640 cm^{-1}, i.e., a value characteristic of COO^- groups, and the complex band in the 2500-3600-cm^{-1} range is decreased, in agreement with the disappearance of the broad ν_{OH} band due to H-bonded COOH groups. At pH 11, relative decreases in the 3100–3600-cm^{-1} range (NH_3^+ stretching), at ca. 1250 cm^{-1} (sym NH_3^+ deformation), and at ca. 800 cm^{-1} (NH_3^+ rocking) agree with the deprotonation of NH_3^+. On the other hand, increases in the 1100–1150-cm^{-1} range (C–N stretching vibrations of aliphatic primary amine) and the trace of a weak doublet in the 3300–3500-cm^{-1} range on the broad ν_{OH} band argue for the appearance of free NH_2 groups. The increase observed at ca. 1420 cm^{-1} remains unexplained.

The acid strength ($pK^a_{1/2} = 9.8$ at $\bar{\alpha} = 1.19$) of glucosammonium residues located within the NDHEP chain may seem rather high in regard to glucosammonium groups in glucosamine ($pK^a_{1/2} \sim 7.8$[18]) and glucosammonium residues in chitosan ($pK^a_{1/2} \sim 5.6$) or in methyl diamino-β-chitobiose ($pK^a_{1/2} \sim 6.8$).[19] However, protonated polymeric bases are generally stronger acids than lower molecular weight analogues. The difference has been attributed to the high positive electric potential of polycations that attracts hydroxyl ions.[16] In the case of NDHEP, the closest neighbor of NH_3^+ is negatively charged and the polyion has an average negative electric potential, thus explaining the weaker acidity of NH_3^+ groups that ionize far above COOH groups. This feature agrees with the fact that acid–base reactions of glucosamine residues are well monitored by CD. However, further investigations are necessary to correlate the CD changes to chromophores and to chemical modifications.

3. Optically Active Chromophores in NDHEP and HEP. A few years ago, we proposed that, in the case of nonstereoordered optically active polyelectrolytes, optical activity changes observed when changing pH or ionic environment depend primarily on chemical modifications of optically active chromophores, in particular ionization, and/or secondary effects of chemical modifications on chromophores.[20] Conformation effects are believed to give minor contributions only, if any, and discrepancies between optical activity changes and chemical composition, sometimes observed, are assigned to closest neighbor interactions.[21]

On the basis of recent papers dealing with glycosaminoglycans,[22] it seems reasonable to assume that, in first approximation, optical activity of this type of polymer and especially of heparin derivatives is linearly related to the composition in saccharidic repeating units. However, this was assumed to be true to evaluate the $pK^a_{1/2}$ of uronic acids in glycosaminoglycans[22] and to discuss chiroptical properties of sodium and calcium salts of heparin.[6]

3.1. Primary Amine Chromophore in Desulfated Heparin. In the $1.00 < \bar{\alpha} < 1.38$ range, only the primary amine groups of glucosamine residues undergo chemical modification from NH_3^+ to NH_2 as previously discussed. It is well-known that the $n \rightarrow \sigma^*$ Cotton effect due to the pair of nonbonding electrons of the nitrogen atom in amine chromophores is usually observed in the 190–220-nm spectral range, depending on substituents.[23] It has been further shown that the corresponding CD band vanishes and is shifted to the far-UV upon protonation in acidic medium.[24]

In order to visualize the chiroptical behavior of glucosamine residues in NDHEP, the effect of protonation on CD spectra of model compounds I and II has been investigated. Figure 5 shows that the low molecular weight model compound exhibits very weak ellipticity in acidic medium while the edge of a large negative band below 200 nm is detected at neutral pH when the compound is deprotonated. Figure 6 shows data obtained for chitosan. In this case, a negative CD band is clearly observed at 210 nm in the protonated form. This band, similar to that detected in *N*-acetyl sugars,[25] can be assigned to residual *N*-acetyl groups present in commercial samples of chitosan.[26] CD measurement at complete deprotonation of

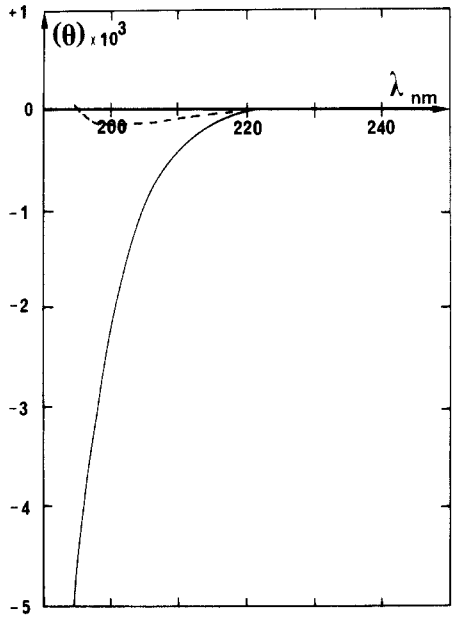

Figure 5. CD spectra of salt-free solutions of methyl diamino-β-chitobiose in the acid form (- - -) and the amine form (—) (T_N = 3.43 × 10^{-3} N, l = 0.01 dm, room temperature).

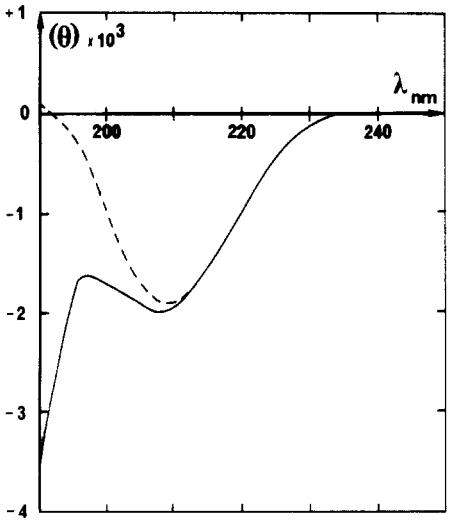

Figure 6. CD spectra of salt-free solutions of chitosan in the acid form (- - -) and at half-neutralization (—) (T_N = 1.73 × 10^{-3} N, l = 0.02 dm, room temperature).

chitosan was not possible because of gelation. However, data at half-neutralization clearly show the appearance of a strong negative CD contribution as in the case of the chitobiose derivative.

On the basis of these characteristics, the CD contribution of the primary amine chromophore in NDHEP can be evaluated as the difference between spectra at NDHEP at $\bar{\alpha}$ = 1.38 (contributing NH_2 form) and at $\bar{\alpha}$ = 1.00 (noncontributing ammonium form). A negative CD contribution is obtained (Figure 7) whose molar characteristics are similar to those of neutral glucosamine residues in model compounds as shown in Table II.

Therefore, it is definitively concluded that CD changes for $\bar{\alpha} > 1.00$ detected in the CD titration curve of NDHEPH (Figure 3) are effectively due to deprotonation of glucosammonium residues.

It is noteworthy that combination of N-desulfation and CD titration of resulting N-desulfated heparinic acid provides a means for determining the functional composition of parent heparin. $\bar{\alpha}_1$, 1.00, and $\bar{\alpha}_2$ being the $\bar{\alpha}$ values at the breaks detected in the CD titration curve of

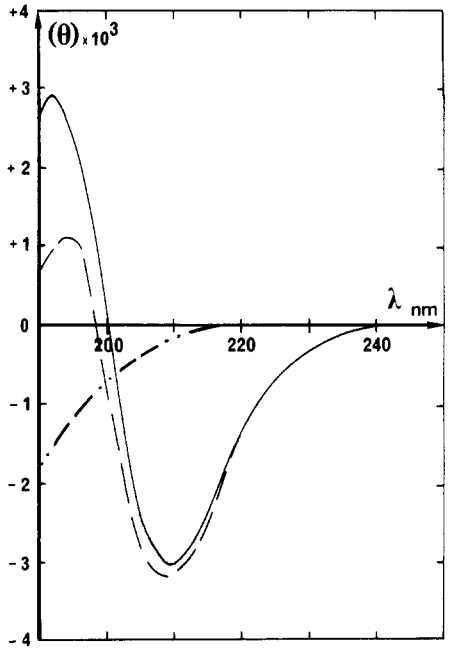

Figure 7. Dichroic spectra of salt-free solutions of NDHEP at $\bar{\alpha}$ = 1.00 (—) and $\bar{\alpha}$ = 1.38 (- - -) (T_N = 2.30 × 10^{-3} N, l = 0.02 dm). The dash-dot line corresponds to the CD contribution of primary amine chromophores in N-desulfated glucosamine residues.

Table II
Molar Ellipticity Contribution due to Glucosamine Moieties in Methyl Diamino-β-chitobiose (I), Chitosan (II), and NDHEP ($\bar{\alpha}$ = 1.38) at Different Wavelengths

	I	II	NDHEP ($\bar{\alpha}$ = 1.38)
T_N × 10^3 M	3.43	1.73	0.89
λ = 195 nm	−1900 ± 100	−1800 ± 200	−1300 ± 300
λ = 200 nm	−870 ± 50	−800 ± 100	−720 ± 150
λ = 205 nm	−320 ± 20	−300 ± 50	−320 ± 100
λ = 210 nm	−130 ± 20	−100 ± 50	−100 ± 100

NDHEPH, percentages of charged groups can be readily deduced from the following relationships:

$$\% \ OSO_3^- = 100 \times (\bar{\alpha}_2 - 1 + \bar{\alpha}_1)/(2\bar{\alpha}_2 - 1)$$

$$\% \ NHSO_3^- = 100 \times (\bar{\alpha}_2 - 1)/(2\bar{\alpha}_2 - 1)$$

$$\% \ COO^- = 100 \times (1 - \bar{\alpha}_1)/(2\bar{\alpha}_2 - 1)$$

For the sample of heparin used in this work, data in Figure 3 lead to 48.3% OSO_3^-, 21.6% $NHSO_3^-$, and 30.1% COO^- groups. These values are in good agreement with those deduced from data reported in the literature,[3] 48.1%, 23.0%, and 28.9%, respectively.

3.2. Sulfamate Chromophore in Heparin. CD contributions of carboxylic pyranoside and *N*-acetylglucosamine residues have been largely described in the literature on glycosaminoglycans.[22] However, no data are available on contributions assignable to sulfamate chromophores in heparin. So far, only the presence of a weak UV absorption band around 190 nm has been reported for cyclohexylsulfamic acid.[27] In order to clear up this point, the CD of N-sulfated glucosamine (III) has been investigated in water (Figure 8). A rather large positive CD band centered at 192–193 nm was found which is independent of pH and counterion as is generally the case for strong acids. Therefore, one can already conclude that sulfamate chromophores may contribute to the CD of heparin in the 190–210-nm spectral range.

As discussed previously, protonated glucosamine residues do not contribute to the CD of NDHEP at $\bar{\alpha} \leq 1.00$.

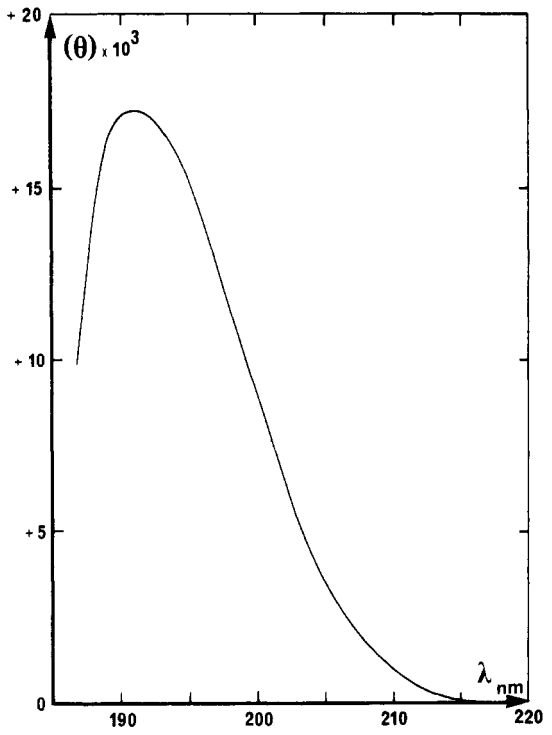

Figure 8. Dichroic spectrum of a salt-free solution of 2-deoxy-2-sulfamino-D-glucose (T_N = 1.24 × 10^{-3} N, l = 0.1 dm, room temperature).

Accordingly, differences between HEPNa and NDHEPNa CD spectra can be considered to reflect the contribution of N-sulfated glucosamine residues if one assumes that contributions of the other chromophores present in both macromolecules are identical. This situation is realized by using solutions of HEPNa and NDHEPNa with the same concentration of COO^- adjusted by conductometric titrations. Results are given in Figure 9. In spite of poor accuracy, a positive CD contribution is clearly detected, in agreement with characteristics of the model compound. Therefore, it is concluded that N-sulfated chromophores present in heparin are optically active. The contribution at 210 nm is weak but it becomes significant at 200 nm and below.

4. CD of Sodium and Calcium Salts of NDHEPH. CD spectra of NDHEPNa and NDHEPCa are presented in Figure 10. Spectra of the two ionic forms of NDHEP are slightly different in magnitude at 210 nm. The crossover wavelength, located at 200 nm for NDHEPNa, is blue shifted to 199 nm for NDHEPCa. For comparison, corresponding data for HEPNa and HEPCa are 202 and 205 nm, respectively.[6] In order to investigate the possible relation between differences in CD spectra and affinity for Ca^{2+} ions, the effect of $CaCl_2$ on the CD of NDHEPNa and that of NaCl on the CD of NDHEPCa have been studied. Figure 11 shows ellipticity changes at 210 nm observed in both cases. Addition of $CaCl_2$ to NDHEPNa gives rise to a sharp decrease of negative ellipticity that becomes invariant for $Ca^{2+}/Na^+ > 5$ (equiv/equiv). In contrast, addition of NaCl to NDHEPCa causes much smoother ellipticity changes, and Na^+/Ca^{2+} ratios higher than 50 are required to reach the value characteristic of NDHEPNa. Except magnitudes, these features are similar to those reported for heparin[6] and considered to reflect the rather strong chemical interactions between carboxylate groups and divalent ions.[6] The fact that N-desulfated heparin behaves similarly to heparin, insofar as salt-induced CD changes are concerned, strongly suggests that NDHEP also has affinity for alkaline-earth cations. This finding dis-

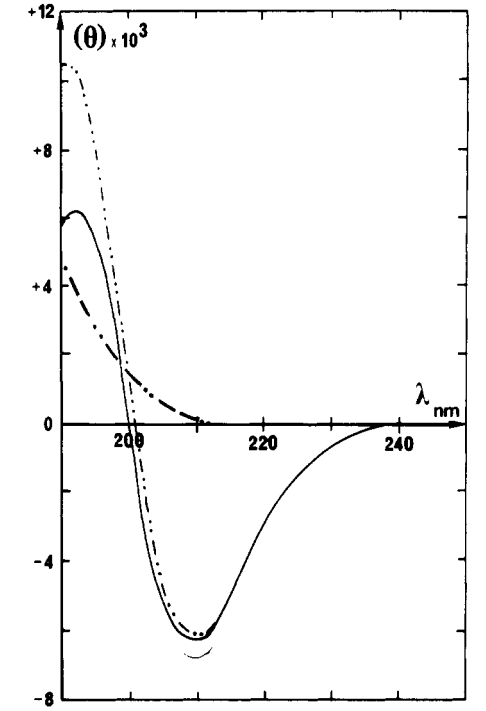

Figure 9. Dichroic spectra of salt-free solutions of HEPNa (··–) (T_N = 3.94 × 10^{-3} N, T_{COOH} = 1.23 × 10^{-3} N) and NDHEPNa (—) (T_N = 2.30 × 10^{-3} N, T_{COOH} = 1.23 × 10^{-3} N) (l = 0.02 dm, room temperature). (– – ··) corresponds to the CD contribution of N-sulfate chromophores in HEPNa.

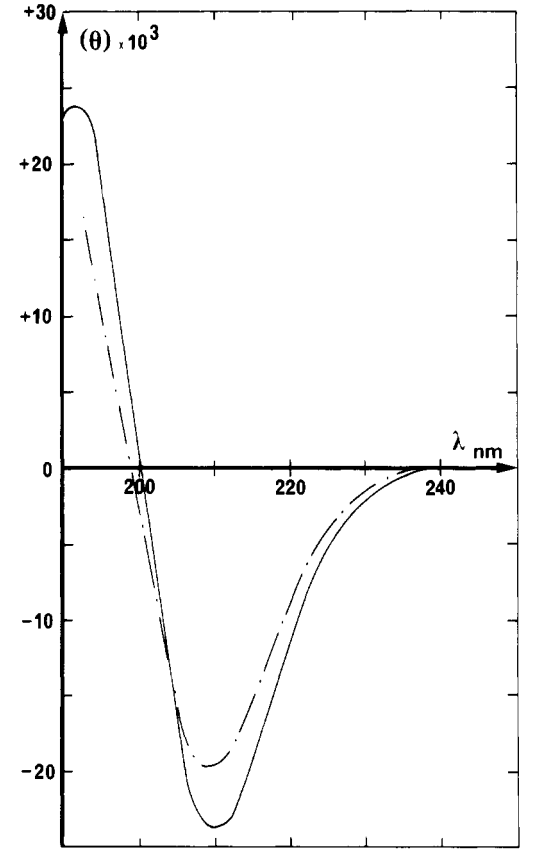

Figure 10. Dichroic spectra of salt-free solutions of NDHEPNa (—) and NDHEPCa (–·–) (T_N = 1.74 × 10^{-3} N, pH 7.5, l = 0.1 dm, room temperature).

agrees with the absence of a counterion effect on the CD of N-desulfated heparin reported in the literature.[7] However, data in ref 7 were obtained at a somewhat lower pH (pH 5.5–6.0) and no experimental details were given

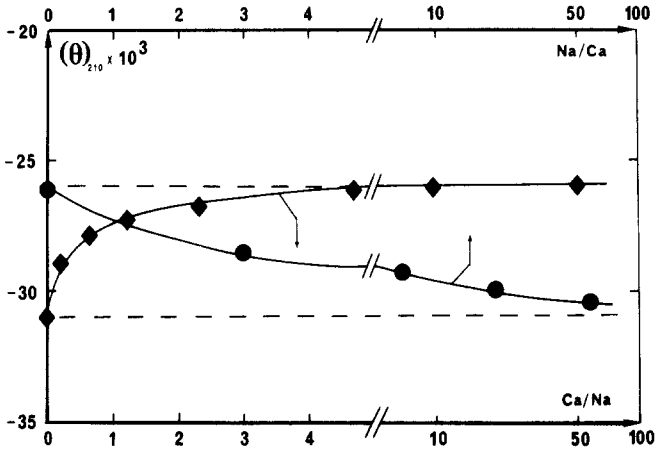

Figure 11. Variations of ellipticity at 210 nm of NDHEPNa (T_N = 2.3 × 10^{-3} N) in the presence of increasing amounts of calcium chloride as defined by the normality ratio Ca^{2+}/Na^+ (♦) and of NDHEPCa (T_N = 2.3 × 10^{-3} N) in the presence of increasing amounts of sodium chloride as defined by the normality ratio Na^+/Ca^{2+} (●).

that may allow more discussion. The difference in magnitude between changes observed for HEP and NDHEP must be regarded as the result of the various modifications occurring at the level of chromophores contributing to the 210-nm CD band and not solely to the absence of N-sulfate groups. Nevertheless, the effects of the zwitterionic structure of NDHEP on chromophores and of the dramatic changes in charge density on complexing properties remain unclear, theoretical support being nonexistent in this domain for systems so complex.

In conclusion, this paper shows that N-desulfation causes not only the loss of one sulfate group but also the pickup of a second sulfate group that ionizes the formed glucosamine residue and turns it into an intramolecular zwitterion stable at neutral. These findings allowed us to propose a simple method to assess the functional composition of heparin on the basis of N-desulfation and CD titration curves. On the other hand, it is clearly shown that CD is primarily sensitive to chemical modification of chromophores. This property allowed us to evaluate the optical activity of amino chromophores in NDHEP and of sulfamate chromophores in heparin. Finally, it is shown that the CD of N-desulfated heparin is cation dependent. Therefore, it is likely that N-sulfate groups do not play the essential role suggested in the literature[7] to account for the affinity of heparin for calcium divalent cations. Investigations of physicochemical properties of fractions and fragments of heparin could provide information in this direction and maybe allow one to conclusively choose between interactions due to polyelectrolyte properties or to the presence of well-defined chelating groups as the source of heparin affinity for divalent cations. Investigations along these lines are under way.

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References and Notes

(1) J. P. Duclos, "L'Hêparine—Fabrication, Structure, Propriêtês, Analyses", Masson, Paris, 1984.
(2) I. Bjork and U. Lindahl, *Mol. Cell. Biochem.* **48**, 161 (1982).
(3) E. Sache, M. Maillard, H. Bertrand, M. Maman, M. Kunz, J. Choay, J. Fareed, and H. Messmore, *Thromb. Res.*, **25**, 443 (1982), and references cited therein.
(4) L. B. Jaques, *Pharm. Rev.*, **31**, 99 (1980).
(5) C. Villiers, C. Braud, and M. Vert, *Carbohydr. Res.*, **83**, 335 (1980).
(6) C. Braud, C. Villiers, and M. Vert, *Carbohydr. Res.*, **86**, 165 (1980).
(7) J. N. Liang, B. Chakrabarti, L. Ayotte, and A. S. Perlin, *Carbohydr. Res.*, **106**, 101 (1982).
(8) Y. Inoue and K. Nagasawa, *Carbohydr. Res.*, **46**, 87 (1976).
(9) C. Braud, M. Vert, and P. Granger, *Carbohydr. Res.*, in press.
(10) Ph. Gatellier, Doctorat 3ème Cycle, Orlêans (France), 1983.
(11) B. Casu and U. Gennaro, *Carbohydr. Res.*, **39**, 168 (1975).
(12) A. S. Perlin, *Fed. Proc. Am. Soc. Exp. Biol.*, **36**, 106 (1977).
(13) L.-A. Fransson, T. N. Huckerby, and I. A. Nieduszynski, *Biochem. J.*, **175**, 299 (1978).
(14) G. Gatti, B. Casu, G. K. Hamer, and A. S. Perlin, *Macromolecules*, **12**, 1001 (1979).
(15) J. R. Helbert and M. A. Marini, *Biochemistry*, **2**, 1101 (1963).
(16) A. Katchalsky, N. Shavit, and H. Eisenberg, *J. Polym. Sci.*, **13**, 69 (1954).
(17) A. D. Cross, "Introduction to Practical Infra-red Spectroscopy", 2nd ed., Butterworths, London, 1964, p 71.
(18) P. W. Kent and M. W. Whitehouse, "Biochemistry of the Aminosugars", Butterworths, London, 1955, p 202.
(19) Unpublished data.
(20) M. Vert, in "Charged and Reactive Polymers—Polyelectrolytes", E. Sêlêgny, Ed., D. Reidel Publishing Co., Dordrecht, Holland, 1974, p 347.
(21) M. Vert, in "Charged and Reactive Polymers—Optically Active Polymers", E. Sêlêgny, Ed., D. Reidel Publishing Co., Dordrecht, Holland, 1979, p 291.
(22) B. Chakrabarti and J. W. Park, *CRC Crit. Rev. Biochem.* **8**, 225 (1980).
(23) J. Huguet and M. Vert, *Eur. Polym. J.*, **12**, 469 (1976).
(24) J. Huguet, *Nouv. J. Chim.*, **3**, 293 (1979).
(25) P. L. Coduti, E. C. Gordon, and C. A. Bush, *Anal. Biochem.* **78**, 9 (1977).
(26) A. Domard and M. Rinaudo, *Int. J. Biol. Macromol.*, **5**, 49 (1983).
(27) A. L. Stone, *Biopolymers*, **10**, 739 (1971).