ACID-BASE AND OPTICAL PROPERTIES OF HEPARIN

J.W. Park[a] and B. Chakrabarti[a,b]

Eye Research Institute of Retina Foundation,[a]
20 Staniford Street, Boston, Mass. 02114,
and Department of Ophthalmology, Harvard Medical
School,[b] Boston, Mass.

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SUMMARY: The apparent pKa value, 5.1, of the carboxyl group of iduronic acid moiety in heparin was determined by circular dichroic and potentiometric titrations. The variations of circular dichroism and optical rotatory dispersion of heparin solution with pH are attributed to the acid-base properties of the carboxyl group rather than to conformational transition. The molecular properties of heparin are discussed in terms of the structure of the molecule.

Heparin is a highly sulfated linear polysaccharide composed of alternating uronic acid and α-D-glucosamine units joined by 1→4 glycosidic linkages. Although the precise structure of heparin is still unknown, studies (1-3) on the polymer have indicated that the glucosamine residues are either N-sulfated (major) or N-acetylated (minor; less than 10% in commercial heparin), and predominantly C-6 sulfated. Most of the uronic acid component is C-2 sulfated α-L-iduronic acid; nonsulfated β-D-glucuronic acid is a minor constituent.

Because of heparin's important biological activities, especially its anticoagulant properties, extensive physicochemical studies have been conducted on this polymer. These include the binding of metal ions (4-6), biogenic amines (7-9), and metachromatic dyes (10,11); hydrodynamic behavior (4,6); and chiroptical measurements (6,9,12). These investigations have shown that the molecular and interaction properties of heparin are critically dependent on pH. Studies (10,11,13) have indicated that carboxyl groups are titrated between pH 3 and 7, but the precise pKa value is still unknown. From the unusual variation of circular dichroism (CD) and optical rotatory dispersion (ORD) properties with pH, Stone (9,10,14) suggested a conformational transition of heparin, on the assumption that the Cotton effect was largely due to amide transition. Since the carboxyl group of

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heparin is one of the chromophores and one potential binding site as well as being involved in the charge distribution of the molecule, the reliable pKa value of the group and its contribution to the overall spectral properties are essential for the interpretation of the pH-dependence of the properties of heparin.

In this study the pH dependence of the CD of heparin has been utilized to determine the pKa value of the carboxyl group and to explore the possible origin of the variation of optical properties with hydrogen ion concentration of the solution.

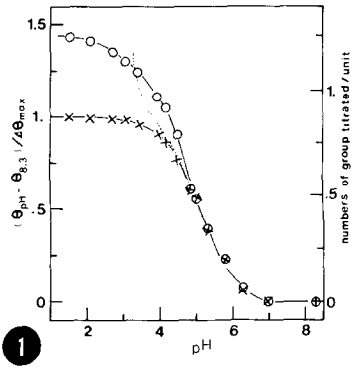
MATERIALS AND METHODS: Sodium salt of heparin (150 to 160 USP units/mg) refractionated from a commercial sample by the use of cetylpyridinium chloride was kindly donated by Dr. G. Armand of Harvard Medical School. The analysis of the sample showed the protein content is less than 0.5% (w/w), and it was used without further purification and fractionation. Stock solution of heparin (5.1 mg/ml) was prepared by dissolving the sodium salt in glass-distilled water. Two solutions, containing 1.70 mg/ml of heparin in 3 x 10^{-4} N NaOH and in 3 x 10^{-2} N HCl respectively, were prepared by diluting the stock solution with NaOH and HCl solutions. A series of heparin solutions with different pH values were obtained by mixing the two solutions in various proportions. CD and ORD were recorded in a Cary 60 and a JASCO UV/ORD-5 spectropolarimeter respectively utilizing 0.2 and 1 cm cells. pH was read at room temperature on a Corning 110 pH meter. The baselines of spectra were corrected for each spectrum but variation of refractive index was not considered. Potentiometric titration was performed with the stock solution against 2.00 x 10^{-3} N HCl with the same pH meter.

RESULTS AND DISCUSSION: The CD and ORD spectra of heparin were measured in the pH range of 1.2 to 8.3. The results are in good agreement with earlier reports by Stone (12,14).

Figure 1 shows the variations of CD ellipticity at 210 and 230 nm, and of potentiometric titration results, with pH.

Generally, the variation of a spectrum with pH reflects changes in species of chromophores due to protonation and deprotonation, and/or their environments due to conformational transition of the molecule. In the absence of any conformational transition, the ellipticity of a CD measurement at a given wavelength is the simple summation of the contributions from all chromophores, and thus it varies linearly with the changes in chemical species. If only one kind of chromophore is changing with pH, the change should follow the equation:

$$pH = pKa - n \cdot \log \left((1 - \alpha)/\alpha \right) \qquad (1)$$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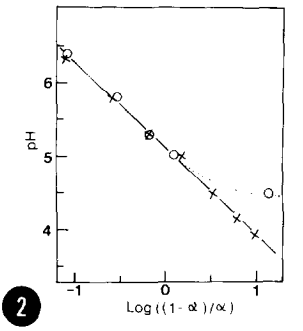

Figure 1. Variation of ellipticity of heparin solution at 210 (x) and 230 nm (o). $(\Delta\Theta)_{max}$ was calculated from $(\Theta_{1.5} - \Theta_{8.3})$ for 210 nm, and from $2(\Theta_{5.1} - \Theta_{8.3})$ for 230 nm. Dotted line is the titration curve calculated by assuming that the average dimeric unit weight (13) is 563. The ordinate scales have been chosen so that the titration curve passes through the same point as the CD curves at pH 5.1. Coincidence of the three curves above pH 5.1 suggests that only one kind of base is acidified in the pH range.

Figure 2. The plots of pH against log $[(1 - \alpha)/\alpha]$ for 210 nm (x) and 230 nm (o). The pH value at log $[(1 - \alpha)/\alpha] = 0$ is the apparent pKa of the acid. Deviation of 230 nm data from linearity is indicative of protonation of another group below pH 5.1.

where α is the fraction of protonated chromophore at a given pH and is related to the ellipticity by the equation, $\alpha = (\Theta - \Theta_{pH \gg pKa})/(\Theta_{pH \ll pKa} - \Theta_{pH \gg pKa})$. n is a constant derived from the activity coefficient; in an ideal system n = 1. However, if any conformational transition is involved, the spectral variation would be much more abrupt than the one expected from equation 1.

The plot of equation 1 from the ellipticity at 210 nm shown in Figure 2 demonstrates that the CD change at 210 nm with pH is indeed due to protonation of a chromophore, the pKa of which is 5.1. The n value, 1.2, is in good agreement with other sulfated glycosaminoglycans (15). Similar analysis for 230 nm ellipticity did not yield a straight line, indicating that the changes at this wavelength cannot be accounted for by a chromophore with a definite pKa value. However, if one assumes that only one chromophore with a pKa of 5.1 is titrated above pH 5.1, one can use twice the ellipticity change up to pH 5.1 as the denominator in the calculation of the value for α of the chromophore. The agreement of

the data for 210 and 230 nm above pH 5.1 in both Figures 1 and 2 shows the validity of this assumption. In addition, the numbers of groups titrated per disaccharide unit support the view.

The chromophore with pKa 5.1 is most likely the carboxyl group of O-sulfated iduronic acid. Mathews (15) showed that the pKa value of iduronic acid (in dermatan sulfate) is 3.93, and that of glucuronic acid (in chondroitin 4-sulfate) is 3.38. The increase of the apparent pKa value of iduronic acid in heparin to 5.1 can be explained by the presence of O-sulfate groups in the component, which produces a strong electrostatic effect from the closely located negatively charged groups. On the other hand, the nonsulfated glucuronic acid component is titrated near pH 3.4, causing a further increase of negative ellipticity at 230 nm in that pH range.

This study suggests that the CD peak of the L-iduronic acid moiety is located near 210 nm, whereas D-glucuronic acid residue has a very small ellipticity at that wavelength. Also, the relatively invariant spectral properties of heparin below pH 3.5 indicates that the dichroism due to sulfate groups remains relatively unaltered upon protonation in those respective wavelength regions.

Morris et al (16) have shown that iduronic acid displays a single CD band above 200 nm, at 210 nm, which has higher ellipticity in an acidic solution without significant change in its position. The same study reported that glucuronic acid showed two CD bands with opposite signs; these were attributed to different rotational isomers. The contribution of the glucuronic acid moiety to the CD spectra of glycosaminoglycans is insignificant near 210 nm (12,17).*

One can estimate the uronic acid composition and the N-acetyl group content of heparin from the known dimeric formula weight. For example, if we use 563 as the dimeric formula weight, we get a value of 85% L-iduronic acid (twice the number of groups titrated up to pH 5.1), which is in good agreement with the generally accepted

*Eyring and Yang (18) suggested that the acidification of carboxyl group in chondroitin 6-sulfate could be accounted for by the CD change of the polymer with pH. However, careful titration studies on the polymer and hyaluronic acid (17) in this laboratory did not yield a direct relationship between CD changes at 210 nm and pKa of the glucuronic acid moiety. On the other hand, the CD change in the 230 to 240 nm region seems to be directly related to the protonation of the carboxyl group.

iduronic acid content. In this case, the changes in molar ellipticity of L-iduronic acid in heparin upon acidification are 1.7 x 10^3 and 0.5 x 10^3 deg•cm^2/dm at 210 and 230 nm respectively. Also, the glucuronic acid moiety changes its molar ellipticity by 1.3 x 10^3 deg•cm^2/dm at 230 nm upon protonation. From the large variation of the 210 nm ellipticity of heparin with pH, one can conclude that the dichroism is mainly due to L-iduronic acid residue rather than the acetoamido group.

This work shows that, whatever the structure and conformation of heparin may be, the variation of optical parameters of heparin with pH is due to the acid-base properties of the carboxyl group rather than to a conformational transition.

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