Effect of Calcium Ions on the Scattering of Light by Heparin

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Abstract □ The light-scattering patterns of sodium heparin under the influence of calcium ions were investigated using unpolarized light of 436 nm. A weight average molecular weight on the order of 17,500 was obtained for samples with varying degrees of sulfation. The interaction constant was found to be influenced by the ionic strength of the solution. At fixed electrolyte concentrations, changes in the second virial coefficient were governed by the charge density of the mucopolysaccharide.

Keyphrases □ Heparin light scattering—effect of calcium^{+2} □ Calcium^{+2}—effect on heparin light scattering □ Molecular weight determination—sulfated heparin

Since its discovery by McLean (1), heparin has been used as a blood anticoagulant. Previous investigations attempted to relate bioactivity with molecular weight from diffusion (2) and sedimentation velocity (3) studies. Using weight average molecular weight, Barlow *et al.* (4) found no correlation with biological activity.

In connecting the anticoagulant properties of heparin with its chemical structure, Foster and Huggard (5) suggested that three effects should be considered: degree and distribution of sulfate groups, molecular size, and shape.

Since highly charged polyions in solution are known to be influenced by the presence of electrolyte (6), the present study was undertaken to examine the scattering patterns of heparin samples, with varying sulfur content and potency, as a function of ionic strength. The data were examined in terms of the interaction constant B relative to bioactivity. Calcium chloride was selected as the damping electrolyte since the heparin–calcium–water model reflects similarity to the blood-clotting system.

MATERIAL

Samples of sodium heparin were obtained[1]. Elemental analysis, on an anhydrous basis, gave sulfur contents of 10.10, 11.92, and 12.99%. Sodium was determined with a Baird Atomic flame photometer, using LiCl and NaCl as internal and reference standards. Triple-distilled sterile water was used as solvent for all solutions whose apparent optical turbidity was found to be on the order of 10^{-5} cm.$^{-1}$. Reagent grade $CaCl_2$ was employed as the damping electrolyte, and all solutions were calculated on the dried basis. Three or four solutions strengths were prepared for each phase of study. Solution clarity was achieved by filtration through 0.2-μ presolution washed Metricel[2] 3-mm. GA-8 disks and subsequently centrifuged for 30 min. at 10,000 r.p.m.[3].

LIGHT SCATTERING

The specific refractive increment, $(n - n_0)c$, where n and n_0 are the refractive indexes for solution and solvent, c is the concentration (g./ml.), and τ (the turbidity) (cm.$^{-1}$) $= 1.17[n^2(Rw/Rc)][\alpha(Gs/Gw)F]$, were obtained with a Brice Phoenix differential refractometer and modified dual photomultiplier-type photometer, model 2000, using 40 × 40-mm. semioctagonal cells and unpolarized incident light of 436 nm. Instrument calibration factors were supplied by the manufacturer with respect to incomplete compensation of refractive effects Rw/Rc and working to opal reference standard α. Photomultiplier responses Gs and Gw were recorded with either no neutral filters F or a combination of filters and α to arrive at the scattering ratio Gs/Gw. Reagent grade NaCl was used in all solutions for dn/dc calibration. The experimentally determined constant, 9.245×10^{-4}, was used to convert readings to refractive index increments and found to be applicable for solutions containing up to 3% sodium heparin.

Table I—Refractive Index Increment and Interaction Constant Data for Sodium Heparin (Sulfur = 12.99%) at 436 nm. and 25°

Solvent	dn/dc(ml./g.) × 10^{-1}	B(mole-ml./g.^{2}) × 10^3
H_2O	1.28	18.00
0.05 M $CaCl_2$	1.29	1.88
0.10 M $CaCl_2$	1.23	1.10
1.00 M $CaCl_2$	1.16	0.22

Weight average molecular weight Mw, defined as $\Sigma N_i M_i^2 / \Sigma N_i \Sigma M_i$, and solution interaction constant B were obtained from the Debye (7) equation $Hc/(\tau_s - \tau_0) = 1/Mw + 2Bc$, in which $(\tau_s - \tau_0)$ is the difference between solution (τ_s) and solvent (τ_0) turbidity reflecting solute or apparent excess turbidity, and c is in terms of grams per milliliter. A plot of the function $Hc/(\tau_s - \tau_0)$ against c when extrapolated to infinite dilution yields the reciprocal molecular weight. The term H is an optical constant equal to $32\pi^3 n^2(dn/dc)^2/3N\lambda^4$, where N is the Avogadro number, λ the wavelength of light in vacuum, and n the refractive index parameter previously described. The B term is the second virial coefficient and reflects deviations from ideality and a measure of the interactions in solution as governed by the thermodynamic properties of the system studied (8). Conditions rendering the sign of this constant positive indicate that net electrostatic charges reside on solute molecules with negative heats of mixing and good solvent properties. Negative values suggest that a high degree of solute–solute interactions is preferred with poor solvation. At $B = 0$, phase separation may ensue. Under these circumstances, for a highly charged macromolecule, the presence of swamping electrolyte can influence the slope of the $Hc/(\tau_s - \tau_0)$ concentration plots.

RESULTS AND DISCUSSION

Heparin was studied under conditions of low (0.05–1.00 M) and high ionic strength, as well as in the absence of electrolyte.

Refractive index increments, given in Table I for heparin, show a distinct dependency on $CaCl_2$ concentration. The data indicate that changes in refractive index, relative to electrolyte concentration changes, are not identical. Previous findings (4) reported constant dn/dc in NaCl.

Average values of $Hc/(\tau_s - \tau_0)$ were plotted against concentration, and lines were fitted by the method of least squares. Each point represents an average of triplicate readings on singularly prepared solutions. Recorder response was found to be within a range of ±1%.

Figures 1 and 2 show all samples per group, irrespective of ionic strength or degree of sulfation, to exhibit close extrapolation. Mean Mw values on the order of 17,600 and 17,200 with a range of ±5%, respectively, were obtained after taking into account a 12% sodium

[1] Eli Lilly & Co. (Lot SO 1292; 138.5 USP units/mg.) and Abbott Laboratories (Lot 807-7199; 157 USP units/mg.).
[2] Gelman Instrument Co.
[3] International Equipment Co., model B-20.

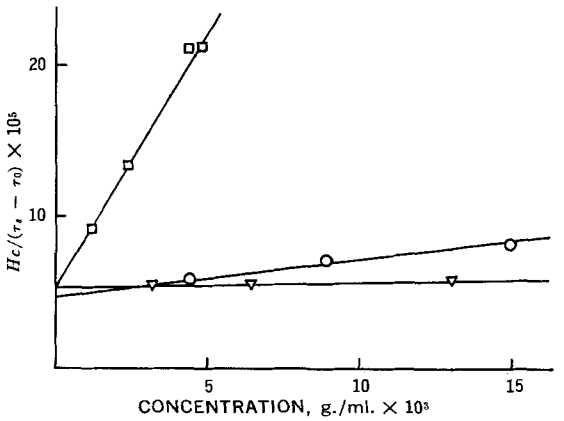

Figure 1—*Effect of ionic strength on the scattering pattern of heparin at 436 nm. and 25°. Key:* □, *H_2O;* ○, *0.1* M *$CaCl_2$; and* ▽, *1.0* M *$CaCl_2$.*

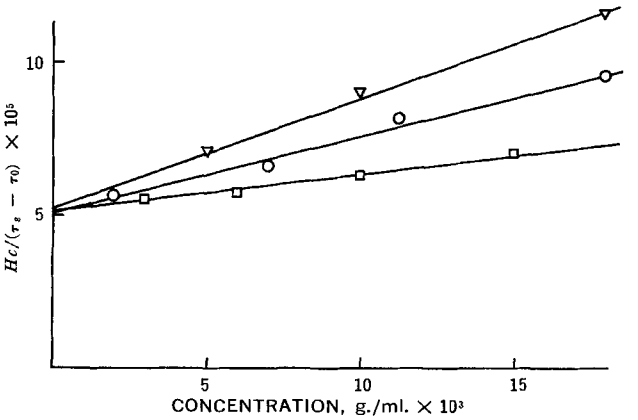

Figure 2—*Scattering of light by heparin samples with varying degrees of sulfation at constant ionic strength* ($\Gamma/2$ = *0.15 $CaCl_2$);* λ = *436 nm.; 25°. Key:* ▽, *12.99% sodium;* ○, *11.92%; and* □, *10.10% sodium.*

contribution. These values are in agreement with those reported from diffusion and sedimentation velocity (2, 3) studies. The results suggest that weight average particle distribution of the polyanion is not influenced by the degree of sulfation in the presence of added electrolyte but is governed by the structural saccharidic backbone. This contention also is supported by the results of Barlow *et al.* (4), who obtained a *Mw* of 16,600 in NaCl for heparin with bioactivities on the order of 5 and 153 units/mg. It appears, therefore, that high ionic strengths are not necessary nor do they greatly influence the extrapolation.

Further scrutiny of the data in Fig. 2, under conditions of constant ionic strength, reveals a progressive decrease in the slopes with diminishing potency. The observed behavior reflects screening of gegenions and seems to denote that with changing polyelectrolyte charge density, the electrical double layer is progressively altered, with less collapse noted in the more highly sulfated species. These findings indicate that the second virial coefficient ($B = 1.83 \times 10^{-3} - 0.68 \times 10^{-3}$ mole-ml./g.^{2}; 157.0–138.5 USP units/mg.) can possibly serve as a physicochemical measure for screening biological potency and may reflect relative differences in potency to a greater degree than weight average molecular weight, which depends on aggregate distribution.

The large positive interaction constant obtained in water is further indication that net electrostatic negative charges reside on the molecules, giving rise to high solute–solvent interactions. Studies (9) with its antagonist, protamine sulfate, showed a negative second virial coefficient on the order of 2.13×10^{-3} mole-ml./g.^{2}.

By contrasting $CaCl_2$ to NaCl, it appears from the present findings that electrolyte valence governs the magnitude of the slopes in accord with theoretical considerations (10). Calcuim chloride at 0.05 *M* was found to influence the slope to the same extent as 0.5 *M* NaCl for heparin samples with reported similar *Mw*. While significant solute–solvent interactions are present in 1.0 *M* NaCl, the present findings in $CaCl_2$ show a drastic decrease in the *B* terms, approximating zero at comparable concentration. Compared to sodium ions, calcium appears to be more strongly bound to heparin, decreasing the ionic character of the mucopolysaccharide to a greater degree. Strong interactions between the two species would be expected to play an important role in the complex anticoagulation mechanism.

Positive slopes noted for all plots also suggest that the particles in solution may exist in the form of flexible chains, as reported by Stones and Moss (11). While heparin has a specific conformation, a degree of flexibility within the molecule can be expected.

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