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Polyelectrolyte Properties of Sodium Hyaluronate. 2. Potentiometric Titration of Hyaluronic Acid

Robert L. Cleland,* John L. Wang,† and David M. Detweiler

Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755.
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ABSTRACT: Titrations of 0.01 *m* glucuronic acid (GA) with NaOH were carried out in cells employing glass electrodes with a saturated calomel electrode (cell A) or a silver chloride electrode (cell B). The dissociation constant at zero ionic strength was given for either cell by pK = 3.23 (±0.02), consistent with previous determinations. Corrections for the liquid-junction potential (cell A) led to the expected behavior of apparent pK with concentration of added $NaNO_3$. Similar titrations of 0.0085 *m* hyaluronic acid (HA) from bovine vitreous humor gave essentially linear plots of apparent pK against degree of ionization α over the range α = 0.3–0.8 in the presence of added salt. Least-squares fits to these plots provided slopes which were fitted better as a function of concentration of added salt by the uniformly charged cylinder model than the infinite line charge model of a polyion. The cylinder radius required to obtain a good fit with the structural charge density is about 10 Å, however, which is larger than the structural radius (4–5 Å) of the charge sites. The discrepancy may be due in part to effects of charge discreteness and low dielectric constant of the polyion. The intrinsic dissociation constant for the polymer was estimated to be pK = 2.9 (±0.1), where the large error estimate reflects uncertainties in extrapolation to α = 0. The difference between polymer (HA) and monomer (GA) pK was attributed to effects of substitution at carbon 4 of the monomer. Although data for electrophoretic mobility at zero polymer concentration are limited for hyaluronate, agreement at one ionic strength (0.1 M) of the surface (ζ) potential calculated from this method and from potentiometric titration suggests that these techniques measure the same potential.

Hyaluronic acid, which occurs in its ionized form in many connective tissues and fluids, is a linear polysaccharide whose repeating disaccharide unit (see Figure 1, paper 1[1]) consists of D-glucuronic acid (β-linked at carbons 1 and 4) and *N*-acetyl-D-glucosamine (β-linked at carbons 1 and 3).[2] At complete ionization of the COOH groups on the glucuronic acid residues, charges occur regularly on every second glucoside and the average charge spacing approximates the length of the disaccharide unit, about 1 nm.

The thermodynamic properties of polyelectrolyte solutions have been extensively studied theoretically; in this work we investigate treatments which model the polyion as a smeared-out charge on an infinite line[3] or rigid cylinder[4,5] or as a flexible polymer chain with discrete charges.[6] In all of these treatments the chain may be characterized by a dimensionless charge density parameter ξ

$$\xi \equiv e^2Q/DkTL \qquad (1)$$

where e is the electron charge, Q is the number of (electron) charges in length L, D is the bulk solvent dielectric constant, k is the Boltzmann constant, and T is the absolute temperature. For monovalent counterions the value ξ = 1 has been sometimes regarded (see ref 3 and citations therein) as a critical value, above which counterion condensation occurs in some fashion near the polyion and below which the latter may be regarded as fully ionized. The value of ξ for the hyaluronate polyion has been taken previously[1] to be 0.70, which is below the critical value. The polymer can, therefore, be tentatively assumed to be fully ionized in the Debye–Hückel sense.

Various experimental features of hyaluronate behavior agree rather well with this assumption in terms of the line charge theory.[3] These include data for the enthalpy of mixing of NaCl and hyaluronate[7] and for activity coefficients in hyaluronate solutions.[8,9] The assumption of complete ionization at $\xi < 1$ has been questioned in a recent discussion of activity coefficients in solutions of polyacrylate copolymers of low charge density,[10] a point which will be dealt with in our discussion.

In the present work the potentiometric titration behavior of hyaluronic acid is examined experimentally and the results are investigated in terms of the available theoretical models as applied to this experimental technique. The most extensive previous investigation by this method was that of Laurent.[11]

Experimental Section

Materials. A crude sample of bovine vitreous humor hyaluronate (K^+ form, Nutritional Biochemical Corp.) weighing 6.14 g was dissolved in 500 cm^3 of deionized water containing (as in all solutions of polymer) 1 mg cm^{-3} of 5,7-dichloro-8-quinolinol (Eastman Kodak Co.) as preservative. The soluton, after dialysis for 48 h against deionized water, was treated with 5.0 g of kaolin (technical grade, washed and ignited, J. T. Baker Co.) for 30 min with stirring and 40 h unstirred, at room temperature. The suspension was clarified by centrifugation at ca. 10^4g for 15 min to give a supernatant containing about 0.08 mg cm^{-3} of protein contaminant. The supernatant (about 400 cm^3) was dialyzed against approximately 5 kg of stirred deionized water for 5 days

† Present address: Department of Biochemistry, Michigan State University, East Lansing, MI 48824.

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with two changes of water. The dialyzed solution was passed through an ion-exchange column (Dowex 50W-X8 H^+ form) to convert the hyaluronate salt to hyaluronic acid. The resulting solution contained 4.0 mg cm^{-3} of hyaluronate (as Na^+ salt) and had a limiting viscosity number $[\eta] = 354$ cm^3 g^{-1} in 0.1 M HCl, corresponding to a weight-average molecular weight[12] of 2.4×10^5 (as Na^+ salt).

D-Glucuronic acid was a commercial grade (Calbiochem, catalog no. 3473), which was found by titration with standard NaOH to have 0.954 equiv of acid per mole (194.1 g) weighed. Sodium hydroxide solutions were prepared from the 50% (w/w) reagent, which was centrifuged at 2000*g* for 10 min to remove suspended insoluble Na_2CO_3, and diluted with CO_2-free water prepared by boiling distilled water for 10 min and cooling in the absence of air. Solutions were stored in polyethylene bottles sealed with rubber stoppers through which were inserted drying tubes containing Ascarite (A. H. Thomas Co.) to prevent contamination with atmospheric CO_2. The diluted solutions were standardized with weighed samples of potassium hydrogen phthalate (reagent grade, Fisher Scientific Co.) dried at 110 °C and assumed pure. Other analytical procedures used in this work have been described previously[13] and include our modifications of standard methods for protein and hexuronic acid analysis.

Potentiometric Titrations. Measurements of pH or cell potential (in mV) were performed with a battery-operated potentiometric pH meter (Radiometer, Model PHM 4). Two kinds of electrochemical cells were used: cell A: glass electrode (G202C)|test solution|saturated KCl|calomel (K401); cell B: glass electrode (G202C)|test solution|AgCl|Ag (P501), where the Radiometer electrode numbers are given in parentheses. The test solutions were contained in a thermostated reaction vessel (Bolab, Inc.) with an outer jacket through which circulated water thermostated at 25.0 (±0.1) °C.

The initial charge, usually about 5 cm^3, of sample for titration or for standardizing electrodes, was pipetted (or weighed, when containing polymer) into the solution chamber. The electrodes were inserted, along with capillary tubes used for bubbling prepurified nitrogen to stir the solution and maintain a CO_2-free atmosphere, the tip of a micrometer-driven microburet (Manostat Corp.), and a magnetic stirring bar to provide supplemental stirring. Spaces around the electrodes and glass tubes were packed with a moldable nondrying putty. As determined by the time required to reach a constant voltage (pH) reading, about 1 h was allowed for initial equilibration of the electrodes with solutions containing polymer or about 10 min with buffers or glucuronic acid. An additional 10 min was allowed after each addition of titrant (NaOH, $NaNO_3$, NaCl solution, or water). Before each titration electrodes were standardized with buffers at 25 °C as follows.

Cell A. Standardization was carried out with 0.01 *m* HCl, 0.09 *m* KCl (pH 2.098 on the National Bureau of Standards practical scale, hereafter called the NBS scale[14]). To correct for error in the change of voltage with pH due to nonideality of electrode behavior, a second buffer containing 0.025 *m* KH_2PO_4, 0.025 *m* Na_2HPO_4 (pH 6.865 on the NBS scale at 25 °C) was used. The correction was performed in practice by use of the instrument's temperature compensation control to balance the potentiometer set at the latter pH value. Other buffers (such as 0.05 *m* potassium hydrogen phthalate, pH 4.008 at 25 °C on the NBS scale) were then checked and found to read correctly within 0.005 pH unit. All buffers were made with reagent grade compounds (J. T. Baker Co.) and distilled water.

Cell B. Cell potential E was measured at 25 °C for 0.01 *m* HCl daily before each titration was performed. The standard cell potential $E°$ (in mV) was redetermined from E for each run by use of the Nernst equation

$$E° = E + b \log a_{HCl} \tag{2}$$

where log denotes base 10 logarithms throughout and a_{HCl} is the HCl activity

$$a_{HCl} = \gamma_H m_H \gamma_{Cl} m_{Cl} = \gamma_\pm^2 m_H m_{Cl} \tag{2a}$$

where m_i is molality, γ_i is the activity coefficient on the molal concentration scale, and $\gamma_\pm$ is the mean ionic activity coefficient. Values of b for a given electrode set were determined (and checked at intervals) from the slope of a calibration plot of E against log a_{HCl} for a series of seven HCl solutions between 10^{-3} and 0.1 *m*. Values of $\gamma_\pm$ were taken from the literature.[15] Resulting values of b were within 1% of the ideal value $b = RT/F = 59.16$ mV, where R is the molar gas constant and F the molal Faraday constant.

Treatment of Experimental Data

The system considered is defined in terms of the following components, having molality m_i or molar concentration C_i: 1, the solvent, H_2O; 2, the acid to be titrated, composed of identical acidic sites HA, where HA represents the ionizable COOH group of D-glucuronic acid (or its residue in the polymer); 3, the added electrolyte NaY, where Y^- is Cl^- or NO_3^-; 4, the titrant base, NaOH. The molality m_2 (or molar concentration C_2) refers to moles of monomer (disaccharide repeat units of the polymer) and hence moles of HA.

The degree of neutralization ν is defined by $\nu \equiv m_4/m_2$. The degree of ionization α was calculated from the condition of electrical neutrality expressed in terms of molalities m_i of the various ionic species

$$\alpha \equiv m_A/m_2 = (m_{Na} + m_H - m_Y - m_{OH})/m_2 = \nu + m_H/m_2 \tag{3}$$

where A^- is the carboxylate anion. The final equality results from stoichiometric relations $m_{Na} = m_3 + \nu m_2$, when complete ionization of hyaluronate is assumed, and $m_Y = m_3$, with $m_{OH} = 0$ to a sufficiently good approximation in the pH range of interest.

The calculation of α thus requires estimates of m_H based on experimental pH readings. For cell B this requires values of $\gamma_\pm$ for HCl, according to eq 2 and 2a. For electrolyte solutions containing very dilute HCl mixed with NaCl or buffers (such as glucuronic acid and its salt) and having total ionic strength I, Bates[16] suggested that this coefficient could be approximated by γ_0, the limiting value at zero HCl concentration of $\gamma_\pm$ (for HCl) in an NaCl solution of ionic strength I. Bates used the Debye–Hückel formula for $I < 0.1$ M

$$g_j \equiv -\log \gamma_j = AI^{1/2}/(1 + \rho_j I^{1/2}) \tag{4}$$

where we take the constant $A = 0.509$ to calculate γ_0 with $\rho_0 = 1.65$ at 25 °C. In solutions containing glucuronic acid, $I = C_3 + \alpha C_2$.

For solutions containing hyaluronic acid the effect of the ionized polymer on $\gamma_\pm$ of HCl was assumed to be identical with its effect on $\gamma_\pm$ of NaCl. As shown in the Appendix, experimental data[8] for NaCl in solutions of fully ionized hyaluronate can be fitted by the additivity rule

$$g_j = g_{js} + g_{jp} \tag{5}$$

where g_{js} represents the effect of salt as given by eq 4 with an appropriate value of ρ_j, $I = C_3$, and g_{jp} represents the effect of the polyion in the form

$$g_{jp} = -\log\left(\frac{1 + \phi_p' X}{1 + X}\right) \tag{6}$$

$$X \equiv m_A/m_3 = \alpha m_2/m_3 \tag{7}$$

To allow for the dependence of ϕ_p' (defined in Appendix) on α, we adopt the approximate expression of the line charge theory[3] $\phi_p' = 1 - \alpha\xi/2$ and set $\xi = 0.54$ for this purpose from the empirical result (see Appendix) that $\phi_p' = 0.73$ at $\alpha = 1$.

For cell A, conversion of the experimental reading of pH $\equiv$ pH(*X*) to $pm_H \equiv -\log m_H$ is more complicated due to the existence of a liquid-junction potential $E_j(X)$ at the boundary test solution|saturated KCl. When standardi-

Table I
Comparison of Calculated and Experimental Values of $\bar{g}_H$

			$\Delta\bar{E}_j$[b]		$\bar{g}_H$			
					X = Na		X = K	
m_{HCl}	m_{XCl}	g_H[a]	X = Na	X = K	calcd	exptl[c]	calcd	exptl[d]
				$pm_H = 2$				
0.01	0.03	0.064	0.010		0.074	0.078		
0.01	0.04	0.068		-0.006			0.062	0.068 (0.065)
0.01	0.09	0.083	0.000	-0.013	0.083	0.078	0.070	0.093 (0.080)
0.01	0.19	0.097	-0.011	-0.020	0.086	0.078	0.077	0.087 (0.081)
0.01	0.49	0.097		-0.032			0.065	0.084 (0.078)
				$pm_H = 3$				
0.001	0.039	0.064	0.004		0.068	0.073		
0.001	0.049	0.068		-0.007			0.061	0.058 (0.062)
0.001	0.099	0.083	-0.006	-0.016	0.077	0.073	0.067	0.087 (0.076)
0.001	0.199	0.097	-0.017	-0.026	0.080	0.073	0.071	0.082 (0.072)
0.001	0.499	0.097		-0.029			0.068	0.071 (0.068)

[a] The values of g_H were calculated from eq 4 with $\rho_H = 2.96$, as suggested by Kielland,[19] for $I < 0.1$ M; $g_H = 0.097$ for $I > 0.1$ M (see text). [b] Values of $\Delta\bar{E}_j$ were calculated as described in the text with the standardizing buffer taken as given in footnotes *c* and *d*. [c] Experimental values from eq 11 of ref 17; standardizing buffer: 0.025 *m* KH_2PO_4, 0.025 *m* Na_2PO_4; calculated value of $\bar{E}_j(S) = 0.032$ (in pH units). [d] Experimental values from mean values in Table I of ref 18; values in parentheses are for KNO_3 in place of XCl; value of $\bar{E}_j(S)$ taken as 0.050 (in pH units) within 0.01 pH unit of buffers a and b of ref 18.

zation is performed as in our experiments, the pH readings may be interpreted in terms of an operational coefficient $\bar{g}_H$ defined by[17]

$$\bar{g}_H \equiv pH(X) - pm_H = g_H + \Delta\bar{E}_j \quad (8)$$

where $\Delta\bar{E}_j \equiv [E_j(X) - E_j(S)]F/(RT \ln 10)$ (in pH units) and $E_j(S)$ is the liquid-junction potential for the standardizing buffer. Hedwig and Powell[17] and McBryde[18] reported calibrations of cells like our cell A with solutions of known m_H in NaCl[17] or KCl[18] from which the experimental values of $\bar{g}_H$ in Table I were obtained. While no reliable method exists for accurately calculating either g_H or $\Delta\bar{E}_j$, the following procedure reproduces reasonably well these experimental values. The single-ion activity coefficient g_H has been estimated from eq 4 with the ionic diameter a_H = 9 Å suggested by Kielland,[19] so that $\rho_H = 0.329 \times 9 = 2.96$ at 25 °C. At $I > 0.1$ M this procedure overestimates g_H, as judged by comparison of calculated and experimental[20] values of γ_0 (with Kielland's estimate of γ_{Cl}). In this range of I, g_H was taken to be 0.097, as suggested by calculations from experimental γ_0 at I = 0.1, 0.2, and 0.5 M, with γ_{Cl} taken equal to $\gamma_\pm$ for NaCl solutions of equal I (Bates–Guggenheim convention[21]). The liquid-junction potentials may be estimated from the Henderson equation as outlined by Bates.[22] The estimated values are listed for HCl–NaCl and HCl–KCl mixtures in Table I along with the calculated values of $\bar{g}_H$ from eq 8. The agreement with the experimental values is within 0.01–0.02 pH unit over the pH range of interest and is comparable to the standard deviations in pH reported by McBryde for the calibrations.[18]

These calculations suggest that calibration of electrodes with solutions of known m_H provides appropriate corrections for the effects of ionic strength of added salts on g_H and the liquid-junction potential. Such calibration cannot be expected, however, to provide correct values of m_H in solutions which contain polyions. Consider the analogous effect of the polyion on apparent γ_{Na} values in solutions of sodium salts of polyacids. For sodium hyaluronate, for which $\xi < 1$, the sodium ions are assumed to be fully ionized, so that m_{Na} is known. Measurements with glass electrodes specific to sodium ion on hyaluronate solutions without added salt have indicated values of $g_{Na,p}$ in the range 0.10–0.15 relative to calibrations with NaCl solutions of known m_{Na}[8,9] (see Appendix). While these values are uncorrected for effects of $\Delta\bar{E}_j$, it seems unlikely that the latter can account for more than a small fraction of the observed cell potential change at a given m_{Na}. In addition, the observed values of γ_{Na} are reasonably consistent with those expected from the line charge theory, as discussed later. In a mixture of "free" counterions, such as H^+ and Na^+, one would expect similar effects on the activity coefficient of each counterion in the polyion atmosphere, since there is no physical reason to distinguish between them, other than the binding capability of H^+ due to the dissociation equilibrium. Although there are no experimental data available to justify the particular form of eq 6 at the lower effective values of α due to the partial ionization in the titration region, inclusion of the correction g_{jp} seemed preferable to ignoring the polyion effect on g_H completely.

All values of pm_H were calculated from pH(X) readings (cell A) by use of eq 8, with g_H obtained from eq 4 (glucuronic acid titrations) or eq 5 (hyaluronic acid titrations) and $\rho_H = 2.96$, except for $I > 0.1$ M, as noted above. The calculation of $\Delta\bar{E}_j$ was performed with equivalent mobilities $\Lambda°$ from the literature[23] and the value $\Lambda° = 75.1$ cm^2 Ω^{-1} equiv^{-1} for glucuronate.[24] Although the mobility of hyaluronate is ionic-strength dependent in the presence of added salt (see below), $\Delta\bar{E}_j$ is insensitive to the value chosen; for titrations without added salt, Λ was assumed to be given by the line charge theory result.[25]

Substitution of α calculated from m_H by eq 3 into

$$pK' \equiv pH + \log [(1 - \alpha)/\alpha] = -\log (a_H m_A/m_{HA}) \quad (9)$$

then gives the apparent dissociation constant pK' for cell A. For cell B, as shown by Bates,[16] experimental data are conveniently evaluated in terms of an approximate pH

$$pwH \equiv -\log m_H - \log (\gamma_H\gamma_{Cl}) = (E - E°)/b + \log m_{Cl} \quad (10)$$

where the second equality is derived from eq 2. As mentioned above, we assumed that $\log (\gamma_H\gamma_{Cl})$ was equal to γ_0^2 and calculated the latter from eq 4 or, for hyaluronic acid solutions, with the extra term of eq 5. Values of α obtained from eq 3 with m_H calculated from eq 10 were substituted into

$$pK'' \equiv pwH + \log [(1 - \alpha)/\alpha] = -\log (a_H m_A \gamma_{Cl}/m_{HA}) \quad (11)$$

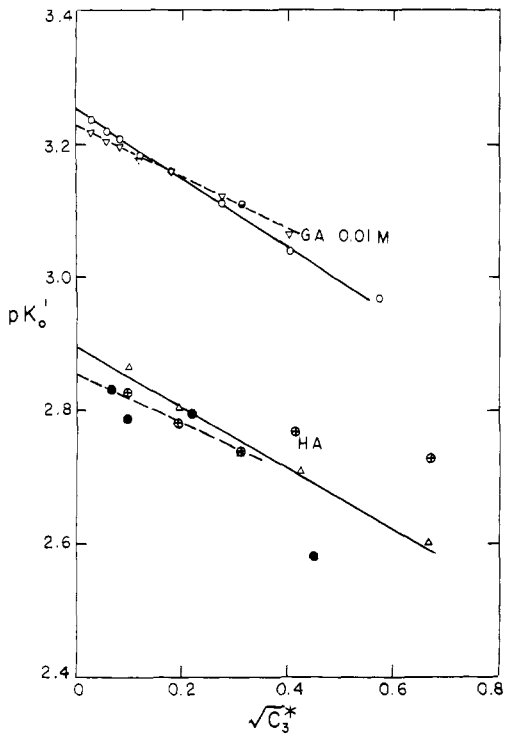

Figure 1. Effect of ionic strength (as $C_3^{*1/2}$) on pK_0', the value of pK' (cell A) at $\alpha = 0$, for 0.01 *m* glucuronic acid (GA) and for 0.0085 *m* hyaluronic acid (HA) at 25 °C. The effects of including corrections for the liquid-junction potential (see text) are shown as follows. Uncorrected points for GA (○) and HA (△, this work) are represented by the solid lines; corrected points for GA (▽) and HA (⊕, this work; ●, data of Laurent[11]) are represented by the dashed lines. The half-filled circle gives the (uncorrected) value reported by Hirsch[28] in 0.1 M KCl.

Comparison of eq 9 and 11 shows that, to the extent our assumptions are valid,

$$pK'' + \log \gamma_{Cl} = pK' = pK + \log (\gamma_A/\gamma_{HA}) \quad (12)$$

where $K = 10^{-pK}$ is the thermodynamic dissociation constant.

Results

Glucuronic Acid. As a check on experimental technique and methods of data treatment, we carried out several titrations, with or without added salt, of D-glucuronic acid. The experimental data included duplicate titrations with NaOH at about 0.01 *m* in cell A and a single titration at 0.01 *m* in cell B. The values of pK' and pK'' calculated from these titrations showed no significant dependence on α (within ±0.01 pK unit) and values were averaged to give $pK'' = 3.22$ (±0.01) in 0.001 *m* NaCl and $pK' = 3.23$ (±0.01) at $m_3 = 0$. In addition, experiments were performed in which neutral salts ($NaNO_3$ in cell A and NaCl in cell B) were added stepwise to partially neutralized samples of glucuronic acid to final salt concentrations m_3 of about 0.5. The values of pK'' showed no dependence on NaCl concentration and averaged to 3.23 (±0.02). The values of pK' showed a marked dependence on ionic strength and are plotted for 0.01 *m* glucuronic acid in Figure 1 against $C_3^{1/2}$ to give a linear plot. The intercept was 3.23 (±0.01), in agreement with titrations at $m_3 \simeq 0$. The slope of the line, which is approximately $-d\gamma_A/dI^{1/2}$ is −0.39. At $I = 0$, $pK'' = pK' - \log \gamma_{HA} = 3.23$ at $m_2 = 0.01$. With the usual assumption that $\gamma = 1$ for uncharged species in dilute solution, the result is $pK = 3.23$ (±0.02).

Hyaluronic Acid. Data for titrations with NaOH were plotted as pK' (cell A) or pK'' (cell B) as a function of α. In Figure 2 are plotted data from a series of titrations with

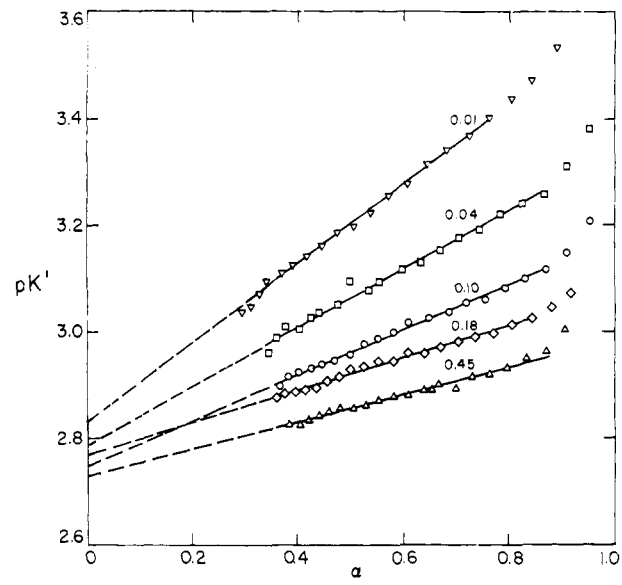

Figure 2. Titration curves (cell A) for 0.0085 *m* hyaluronic acid titrated with NaOH in $NaNO_3$ solutions having the following values of C_3^* ($NaNO_3$ molarity in the corresponding dialysis equilibrium solvent): 0.01 M (▽); 0.04 M (□); 0.10 M (○); 0.18 M (◇); 0.45 M (△). The straight lines represent linear least-squares fits to the linear sections of the data with the parameters and the range of α for the fitted points given in Table II.

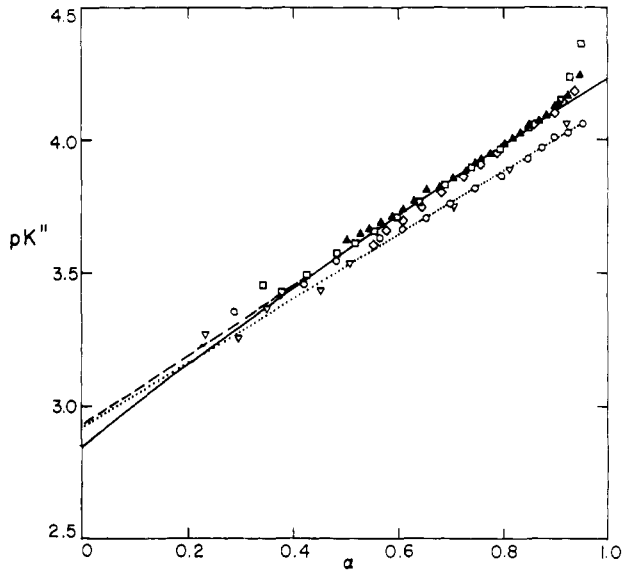

Figure 3. Titration curves (cell B) for hyaluronic acid with NaOH at $C_3^* = 0.001$ M. Points, corrected for dilution as described in the text, are shown for the following values of polymer equivalent molality m_2: 0.0089 (▽); 0.0045 (○); 0.0025 (□); 0.0013 (◇); 0.0010 (▲). Fits to linear sections of the data are shown for 0.001–0.0025 *m* (– – –) and for 0.0045–0.0089 *m* (···). The solid curve is a fit to data calculated from tabulated solutions[5] to the uniformly charged cylinder model appropriate for 0.001 M salt and radius $a = 10$ Å.

cell A at $m_2 = 0.0085$ at concentrations of $NaNO_3$ between 0.01 and 0.45 M. Data shown in Figure 3 represent a series of titrations with cell B at different m_2 from 0.001 to 0.0087 at $C_3^* = 0.001$ M NaCl, where C_3^* is the composition the polymer-free solvent (dialysate) would have at dialysis equilibrium. The actual points in both plots were corrected for dilution of salt and polymer during titration by the following procedure.

The assumption was made, consistent with the Hill–Stigter model of polyion solutions,[26] that C_3^* was the appropriate salt concentration for comparison with theoretical models which neglect polyion interactions; extrap-

Table II
Parameters from Linear Least-Square Fits to Data for Titrations with NaOH

m_2	C_3*[a]	pK_0'[b]	$(\partial pK'/\partial\alpha)_{C_3*}$[b]	α range fitted	no. of points
		Cell A			
0.0085	0.000	2.92 (±0.01)	0.81 (±0.02)	0.25-0.84	38[c]
0.0085	0.000	2.86 (±0.01)	0.84 (±0.02)	0.39-0.93	22
0.0085	0.010	2.82 (±0.01)	0.77 (±0.01)	0.29-0.85	19
0.0085	0.040	2.78 (±0.01)	0.55 (±0.02)	0.33-0.87	19
0.0085	0.100	2.74 (±0.01)	0.43 (±0.01)	0.35-0.87	19
0.0085	0.180	2.77 (±0.01)	0.30 (±0.01)	0.36-0.84	19
0.0085	0.450	2.73 (±0.01)	0.25 (±0.01)	0.38-0.80	17
0.0043	0.000	2.82 (±0.02)	1.04 (±0.04)	0.34-0.58	7
0.0010	0.000	2.77 (±0.01)	1.17 (±0.01)	0.50-0.84	25
0.0010	0.000	2.82 (±0.02)	1.29 (±0.04)	0.45-0.57	8
		Cell B			
0.0089	0.001	2.93 (±0.01)	1.21 (±0.01)	0.35-0.81	5
0.0045	0.001	2.97 (±0.01)	1.13 (±0.02)	0.42-0.90	11
0.0025	0.001	2.93 (±0.01)	1.30 (±0.01)	0.55-0.90	13
0.0023	0.001	2.96 (±0.01)	1.20 (±0.01)	0.49-0.90	10
0.0010	0.001	2.96 (±0.01)	1.29 (±0.02)	0.55-0.90	20

[a] This molar concentration refers to the dialysis equilibrium solvent as explained in the text; the salt is $NaNO_3$ in cell A and NaCl in cell B. [b] The intercept pK_0' and the slope $(\partial pK'/\partial\alpha)_{C_3*}$ are parameters from the least-squares fit over the range of α and with the number of points given. In cell B pK' becomes pK''. Uncertainties given are standard deviations in the fitting procedure only. [c] Three nearly identical titrations are combined for this fit.

olation to infinite dilution of polymer should also be carried out. Values of C_3* were calculated from eq 1-1 with the virial coefficients for salt distribution $A_2 = 0$ and A_1 estimated from a fit of eq 1-11 with $\bar{V}_2'$, the partial molal volume, taken to be 500 cm^3 mol^{-1}, appropriate to a cross-sectional chain radius of 5 Å, as a reasonable fit to the data of Figure 1-3. Since the best empirical fit to the dependence of apparent pK values on salt concentration was given by the numerical solutions of Stigter[5] (see Figure 5 and Discussion), an empirical correction to a single value of $C_3* \equiv (C_3*)_0$ for each titration was made by use of a fit to the theoretical curve of Figure 5 marked S10. Experimental values of pK' (or pK'') were corrected by adding a term δpK defined by

$$\delta pK \equiv pK'(\text{corrected}) - pK' = (\partial pK'/\partial \log C_3*)_\alpha \log [C_3*/(C_3*)_0] \quad (13)$$

A small correction for the effect of C_3* on pK_0' (see eq 16), based on the slope in Figure 1, was also included. These corrections were negligible for titration at $C_3* > 0.01$ M. Points shown in Figures 2 and 3 were corrected by this procedure.

It is perhaps worth mentioning that the Manning–Holtzer formulation[27] could be used in a conceptually equivalent way. As the appropriate concentration variable, the latter authors used log $(C_3 + \alpha C_2/2)$, which is just log C_3* when the Donnan equilibrium expression $A_1 = -1/2$ is used. The correction δpK would then proceed through eq 18 (see below) with an empirical fit of the data to obtain an effective ξ.

A series of titrations without added salt were also carried out with cell A at different values of m_2 between 0.001 and 0.0085. These titrations are not actually salt-free, since leakage from the saturated KCl salt bridge was estimated at 0.0002 M/h, so that C_0 may have reached 0.0005–0.001 M during a long titration. A correction procedure for polymer diultion similar to that described above was carried out by use of a fit to the Lifson–Katchalsky theory (curve LK10 in Figure 6), so that each curve refers to a single polymer concentration C_2 in Figure 4, where the corrected points are plotted.

Duplicate titrations were reproducible only to about 0.01–0.02 pH unit; a discrepancy of 0.02 pH unit suffices to produce the differences in pK' values for the two titrations at $m_2 = 0.001$. A standard deviation in pK' values

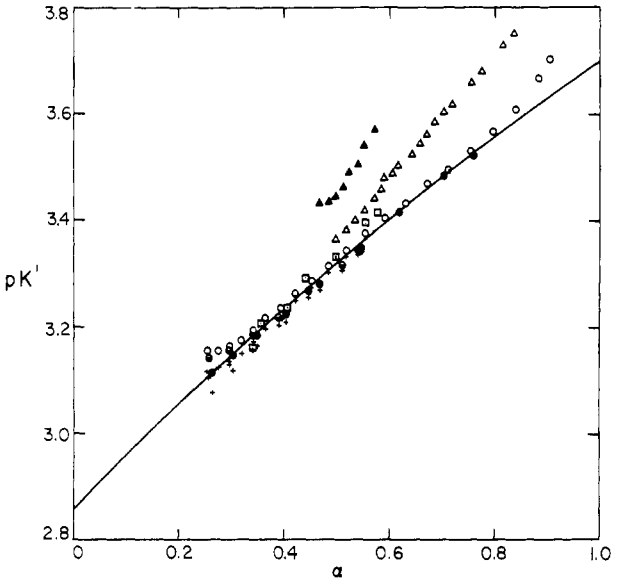

Figure 4. "Salt-free" titrations curves (cell A) for hyaluronic acid with NaOH at the following equivalent molar concentrations C_2: 0.0085 M (three titrations, ○, ◒, and ●); 0.0043 M (□); 0.001 M (two titrations, △ and ▲). Crosses represent corrections to the data at 0.0085 M to allow for the effect of the polymeric N-acetyl groups when the latter are assumed to have dissociation pK = 0.8 (see text). The solid line represents a fit to the data at 0.0085 M by the Lifson–Katchalsky theory with a taken to be 7.5 Å.

of about 0.02 pK unit for midrange values of α is estimated for titrations at $m_2 = 0.0085$, at which most of our data were obtained. The effect of the correction term g_{jp} in eq 5 on calculated values of pK' (and pK'') was significant only for titrations in which $m_2 > m_3$, and then only in the initial stages of the titration., where the correction is of the order of a few hundredths (up to 0.05) pK unit.

As discussed later, the correct procedure for extrapolation of the plots of apparent pK against α to obtain pK_0', the intercept at $\alpha = 0$, is not clear, and extrapolated values of pK_0' are therefore affected by some uncertainty. The plots in Figure 2 at $C_3* \geq 0.01$ M are approximately linear, with positive curvature evident only at $\alpha > 0.9$. Parameters from least-squares linear fits to the data for $\alpha < 0.9$ are given in Table II. At $C_3* = 0.001$ M (Figure 3) and in the "salt-free" titrations of Figure 4, pronounced cur-

vature appears also at values of α below about 0.3 in most titrations. In these cases we regard the fits to linear sections, typically between α = 0.3 and 0.85, as reasonably accurate only for slopes at midrange values of α. Second-degree fits including a term in α^2 increase pK_0' typically by 0.02–0.05 pK unit at C_3^* > 0.01 M and as much as 0.10 pK unit at 0.001 M, without significant effect on the slope at, say, α = 0.6.

The pK_0' values obtained from the linear fits at C_3^* > 0.01 M, as well as those obtained without correction for $\Delta\bar{E}_j$ are plotted in Figure 1. As in the case of glucuronic acid, the intercept at C_3^* = 0, which we call pK, is somewhat smaller; pK = 2.86 (±0.02) (where standard deviations refer only to fitting errors) when $\Delta\bar{E}_j$ is included in the correction. While the absolute values of apparent pK are rather uncertain, these plots should indicate the approximate dependence on C_3^*, which appears to be similar to that for the low-molecular-weight acid.

Discussion

Glucuronic Acid. The estimate pK = 3.23 (±0.02) found here for the acid dissociation constant of D-glucuronic acid may be compared with values reported previously. In Figure 1 is plotted a value (half-filled circle) obtained in 0.01 M KCl by Hirsch,[28] who also reported pK = 3.18 at 20 °C. Kohn and Kováč[29] found pK' = 3.28 (±0.01) at 20 °C for m_2 = 0.003. The latter authors cited three other previous determinations of pK by potentiometry and one study[24] employing both conductometric and potentiometric methods, which gave values of pK ranging from 3.20 to 3.24; one very early conductometric determination[30] gave pK = 3.33 (±0.02). The present determination of pK thus confirms previous findings.

The good agreement between results from cell A and cell B provides support for the correction procedure employed for cell A. While extrapolated values of pK are not very sensitive to the correction term $\Delta\bar{E}_j$, the slope of apparent pK values with $C_3^{1/2}$ is significantly affected. The finding with cell B that no significant change of pK'' occurs with change of C_3 suggests, according to eq 12, that $\gamma_{Cl} \simeq \gamma_A$. The slope of the plot for glucuronic acid in Figure 1 should therefore be similar to that expected for γ_{Cl}. This expectation may be compared with experiment by assumption of one of the single-ion conventions, such as the Bates–Guggenheim convention referred to above, for which $\gamma_{Cl} = \gamma_{\pm,NaCl}$. The change of p$K'$ with $C_3^{1/2}$ shown for the correction with $\Delta\bar{E}_j$ follows that predicted by the latter, consistent with the result for cell B.

Hyaluronic Acid. A suitable starting point for the discussion of potentiometric titration of polyacids is the expression of Overbeek[31] for the apparent dissociation constant of a polymer containing N_2 identical sites of intrinsic acid dissociation constant K

$$pK' = pK + (0.434/N_2kT)(\partial A_{el}/\partial\alpha)_T \qquad (14)$$

where A_{el} is the electrostatic free energy per polyion. As pointed out by Harris and Rice,[6] this relation with pK' defined by eq 9 implies random mixing of charged and uncharged sites and neglect of specific counterion binding. Besides the entropic terms introduced when the latter conditions are not met and the direct electrostatic free energy of charge interactions accounted for by the usual charging process, A_{el} contains, in general, configurational free energy changes induced by charging. When the latter may neglected, A_{el} may be regarded as derived from the (averaged) electrostatic potential ψ_0 at the charged sites and eq 14 becomes

$$pK' = pK_0' + 0.434y_0 \qquad (15)$$

where $y_0 \equiv e\psi_0/kT$. In their treatment of the polyion as a set of discrete charges, Harris and Rice[6] included a free energy term A_a for the formation of ionic atmospheres about these charges, which they took, in the presence of excess salt, to be given by the Debye–Hückel expression for monovalent ions, so that

$$pK_0' = pK + (0.434/N_2kT)(\partial A_a/\partial\alpha)_T = pK - 0.434e^2\kappa/2DkT \qquad (16)$$

where κ is the usual Debye screening parameter, $\kappa = (\lambda\sum_i C_i)^{1/2}$, $\lambda \equiv 4\pi e^2 N_A/10^3 DkT$, N_A is Avogadro's number, and the sum is taken over mobile ion species i. The identical treatment of acids of low molecular weight leads to eq 12 with γ_A given by a form of eq 4. A similar treatment of pK_0' was used by Muroga et al.[32] in their analysis of potentiometric titration data for (carboxymethyl)cellulose in NaCl solution, where the assumption was made that the isolated carboxylate ions in the polymer would have the same activity coefficient as in solutions of monobasic carboxylic acids. The slope of the hyaluronic acid data, as drawn in Figure 1, approximates that for glucuronic acid, suggesting that eq 16 is qualitatively valid.

A. Infinite Line Charge Model. The infinite line charge theory of Manning[3] gives for y_0 for monovalent ionic sites

$$y_0 = -\alpha\xi \ln \kappa^2 = -\alpha\xi \ln (\lambda\sum_i C_i) \qquad (17)$$

Inclusion in A_{el} (and thus y_0) of the Born approximation to the free energy of charging an infinite cylinder of radius a and substitution of eq 17 into eq 15 lead, as shown by Manning and Holtzer,[27] to

$$pK' = pK_0' - \alpha\xi[\log (2\lambda a^2) + \log (C_3 + \alpha C_2/2)] \qquad (18)$$

While the line charge theory is the polyion analogue of the Debye–Hückel treatment and is thus essentially an infinite dilution theory, many of its consequences appear to hold att moderate ionic strengths.[33]

According to eq 18, the slope $(\partial pK'/\partial\alpha)_{C_3}$ is given by

$$(\partial pK'/\partial\alpha)_{C_3} = -\xi[\log (2\lambda a^2) + \log (C_3 + \alpha C_2/2)] - 0.434\xi\alpha C_2/(2C_3 + \alpha C_2) \qquad (19)$$

At the limit C_2 = 0, $(\partial pK'/\partial\alpha)_{C_3}$ plotted against log C_3 should give a straight line of slope $-\xi$. The values given in Table II, as well as those calculated by least-squares fitting of titration data in NaCl soultion reported by Laurent,[11] are plotted in this fashion in Figure 5. When compared to the theoretical result of eq 19 for hyaluronic acid (dashed line of slope $-\xi$ = −0.7), the experimental slope, which corresponds to an effective ξ of 0.3–0.4, is clearly too small. This result is consistent with previous findings that effective ξ values derived from comparison of experimental data with expressions from the line charge theory are smaller than the value deduced from the polymer structure. Such empirical values of ξ include the following: mean activity coefficient of NaCl[8] (ξ = 0.58[34] or 0.54; see Appendix); Na^+ activity coefficient in salt-free solution[9] (ξ = 0.6[35]); enthalpy of mixing with NaCl (ξ = 0.6[7]). This point is discussed further below. In their comparison of eq 18 with experimental titration data, Manning and Holtzer[27] found a similar discrepancy (i.e., low effective ξ) in a plot of $(\partial pK/\partial \log C_3)_\alpha$ against α for (carboxymethyl)cellulose titration data.

B. Uniformly Charged Cylinder Model. As is well-known, inclusion of the effect of the (hard sphere) size of small ions in the calculation of the potential provided an important improvement to the Debye–Hückel limiting law in fitting activity coefficient data for electrolyte salts. The corresponding treatment of the uniformly charged cylinder model for the polyion in the Debye–Hückel lin-

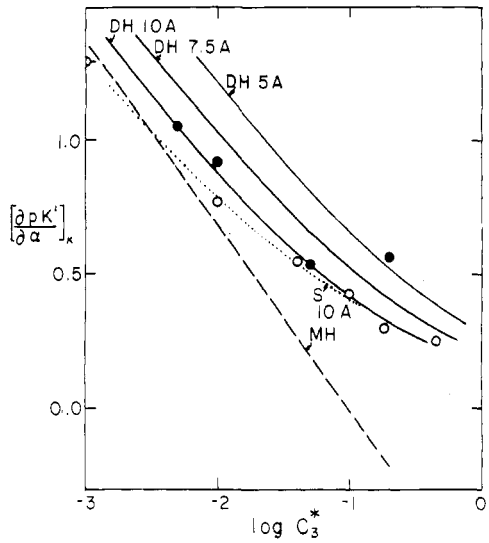

Figure 5. Least-squares fitted slopes of titration curves (pK' vs. α) with NaOH for hyaluronic acid in excess salt as a function of log C_3^*. Experimental points from Table II are shown as open circles (at $m_2 = 0.0085$, except the value at log $C_3^* = -3$ from pK'' data (cell B), which represents extrapolation to $m_2 = 0$). Filled circles are from the data of Laurent,[11] recalculated as for titrations in this work. Theoretical curves are shown ($\xi = 0.7$) for the Manning–Holtzer (MH) infinite line charge model (cylinder radius $a = 10$ Å) and the uniformly charged infinite cylinder model for the linearized (DH at $a = 5$, 7.5, and 10 Å, eq 20 with $b = a$) and nonlinearized[5] (S, $a = 10$ Å) form of the Poisson–Boltzmann equation.

earization approximation to the Poisson–Boltzmann equation was apparently first given by Gorin[36] and later independently by Hill.[37] Their result may be written

$$\frac{\mathrm{p}K' - \mathrm{p}K_0'}{\alpha} = \left(\frac{\partial \mathrm{p}K'}{\partial \alpha}\right)_{x_0} = 2(0.434)\xi\left[\frac{K_0(x_0)}{x_0 K_1(x_0)} + \ln(a/b)\right] \quad (20)$$

where $x_0 = \kappa a$, a is the radius of closest approach to the center of the cylinder of a counterion center, b is the cylinder radius, and $K_0(x_0)$ and $K_1(x_0)$ are modified Bessel functions of the second kind. The same problem has been treated more accurately by carrying out detailed integrations of the nonlinearized Poisson–Boltzmann equation;[5,38] Stigter[5] tabulated his results in a manner which is convenient for calculation of y_0 from arbitary values of ξ and x_0. As pointed out by Stigter, these calculations are stictly valid only for the case of added electrolyte. Lifson and Katchalsky[39] gave an analytical solution for the nonlinearized equation in the salt-free case by assuming a model consisting of an array of uniformly spaced parallel infinite cylinders to represent the polyion solution.

The curves labeled DH in Figure 5 were calculated from eq 20 for $a = 5$, 7.5, and 10 Å with omission of the term $\ln(a/b)$. The dotted line S gives values corresponding to $\alpha = 0.65$ as calculated from the tabulations of Stigter[5] with $a = 10$ Å. The introduction of a specified value of α is required because $(\partial y_0/\partial\alpha)_\kappa$ depends on α, in contrast to the linearized case. As α approaches zero or for large x_0 at all α, the S and DH curves converge.

The uniformly charged cylinder models clearly give a better fit to the *form* of the potential change with salt concentration than does the uniformly charged line model. Numerical agreement is achieved with the former in Figure 5, however, only for a cylinder radius a of about 10 Å with neglect of the term $\ln(a/b)$ in eq 20. A radius of this magnitude is somewhat difficult to reconcile with structural models of hyaluronic acid, however. In previous work a cylinder radius (corresponding to b in the present model) between 3.4 and 4 Å was estimated for the hyaluronate polyion modeled as a cylinder on the basis of (1) the specific volume[1] and (2) small-angle X-ray scattering.[40] Radii in this range were used to fit satisfactorily intrinsic viscosity and sedimentation coefficient data[40] to theoretical calculations based on a cylindrical model of the wormlike chain. A similar radius can be estimated for the charged sites from the atomic coordinates of the carboxylate oxygens.[41] A hydrated radius of about 3 Å for the sodium ion would lead to a structural estimate for a of about 7 Å, provided that no additional water layer hydrates the ionic sites of the polymer. Data on salt exclusion[1] suggest a radius between 4 and 7 Å.

Calculation of the expected $\partial \mathrm{p}K'/\partial\alpha$ from this structural model with eq 20 leads to much higher potentials than those observed experimentally. Inclusion of the term $\ln(a/b)$ leads, especially at high ionic strengths, to higher values at a given b than those calculated in Figure 5 with $a = b$ (see curve DH5 for $b = 5$ Å). It ξ is used as an adjustable parameter, the experimental data can be fitted reasonably well by taking $a = b = 3.6$ Å with $\xi = 0.4$ or $a = b = 5.8$ Å with $\xi = 0.525$. Numerical calculations based on continuum models such as the Poisson–Boltzmann or Navier–Stokes equation can be expected to apply only when particle dimensions are in the "colloidal" range. For example, Stokes' law apparently leads to error in applications to particles of radius less than about 5 Å (ref 23, p 125), so that we must regard numerical results in this size region as semiquantitative.

An error in the assumed value of ξ would result if the Na^+ ions are not completely ionized. As mentioned in the introduction, Kowblansky and Zema[10] made such a proposal to explain activity coefficients smaller than those predicted by the line charge theory for a simple 1:1 salt in solutions of polyacrylate copolymers for which the structural value of ξ was less than unity. They found a good fit to the data with our eq A5 (see Appendix) using experimental values of γ_p, which were also smaller than theoretical predictions. (Note that values of ξ estimated from γ_p would then be *too high*.) As shown in the Appendix, a similarly good fit can be provided for hyaluronate solutions with use of the theoretical value of γ_p from the line charge theory; in fact, reported measurements[8,9] of γ_p, while subject to considerable uncertainty (see Appendix) suggest that Na^+ is at least as highly ionized as expected from theory. Available evidence is therefore in favor of the assumption of complete ionization.

The need to use larger than structural radii to fit titration data is not limited to hyaluronate. Muroga et al.[32] fitted their titration data for (carboxymethyl)cellulose with the approximate structural radius $a = 10.3$ Å. Their theoretical values of pK, which were obtained from numerical solutions[38] of the nonlinearized Poisson–Boltzmann equation, agreed reasonably well with experiment at high α ($\xi < 1$) but showed increasing deviations as α decreased (to $\xi < 1$). These authors discussed a similar effect in the case of coiled poly(DL-glutamic acid). They also found that for helical poly(D-glutamic acid) (charge spacing 20 Å) a radius a of 14 Å was needed to fit titration data rather than the structural value of 9 Å. The uniformly charged cylinder model therefore appears to overestimate generally the surface potential at low charge density, provided counterion binding can be neglected at $\xi < 1$.

The rigid cylinder is admittedly a rough approximation for a flexible polyelectrolyte like the hyaluronate ion. A

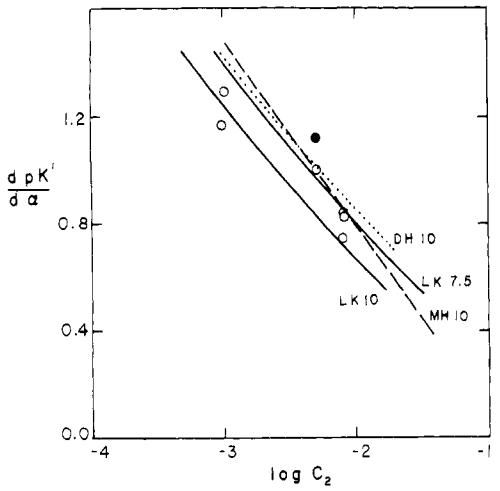

Figure 6. Slopes of "salt-free" titration curves (pK' vs. α) with NaOH for hyaluronic acid of different equivalent molar concentrations C_2 (○, this work; ●, from data of Laurent[11]) at $C_3^* = 0$ (except for salt leak from the calomel electrode). Theoretical curves are shown for the case of no excess salt from the Manning–Holtzer model[27] (MH, a = 10 Å), the Lifson–Katchalsky model[39] (LK, a = 7.5 and 10 Å), and the uniformly charged cylinder model (DH, a = 10 Å, calculated as described in the text).

more realistic model in which the uniform charge density is replaced by a discrete charge distribution might improve this situation. Tanford[42] showed, for the case of a spherical surface, that such replacement led to a lowering of the slope of the titration curve by as much as a factor of 2. Preliminary calculations show that the nearest-neighbor site model of Hill[43] with the screened Debye–Hückel potential interaction between charged sites on the polyion predicts values of $\partial pK'/\partial\alpha$ significantly *smaller* than those in Table II. A more sophisticated model in the spirit of the Harris–Rice treatment[6] allowing for the effect of dielectric constant on charging energy of the polyion, second and higher neighbor effects on the entropy of mixing, and conformational energy effects due to coiling might be expected to improve this situation.

Titrations in the Absence of Salt. The slopes given in Table II for "salt-free" titrations are plotted in Figure 6 as a function of log C_2. Also plotted, for purposes of comparison, are theoretical curves derived from the Lifson–Katchalsky (LK) treatment[39] for a = 7.5 and 10 Å. Values of C_2 are related to a by the parallel-rod model used by these authors. Differentiation at $\alpha = 0.5$ of a plot of values of the potential calculated at several values of α was used to obtain the plotted curves.

The Manning–Holtzer and Debye–Hückel (Gorin–Hill) models are both derived from the linear approximation to the Poisson–Boltzmann equation for added electrolyte. Application to the salt-free case of these models is based on the somewhat arbitrary procedure employed by Manning[3] of considering the mobile counterion as a part of $\sum C_i$ in eq 17, as illustrated by eq 18. Equation 19 has therefore been used with a = 10 Å and $C_3 = 0$ to construct the dashed line MH10 in Figure 6. Similar differentiation of eq 20, with x_0 now a function of α, leads for the DH model with $a = b$ to

$$\frac{d(pK')}{d\alpha} = 0.434\xi\left[\frac{2K_0(x_0)}{x_0K_1(x_0)} + \left(\frac{K_0(x_0)}{K_1(x_0)}\right)^2 - 1\right] \qquad (21)$$

The dotted line in Figure 6 represents the same assumption with regard to the calculation of κ in the salt-free case as that used by Manning. The various theoretical models give reasonable agreement with the experimental data with a radius a similar to that providing a good empirical fit in the presence of excess salt.

Extrapolation to Obtain pK_0'. While the slopes of the titration curves are rather well defined, at least in the middle range of α, the extrapolation to $\alpha = 0$ needed to obtain pK_0' must be regarded as somewhat uncertain. Theoretical treatment of the uniformly charged cylinder predicts that the surface potential (hence pK') is a linear function of α when the linearized approximation to the Poisson–Boltzmann equation (see eq 20) is used. The nonlinearized form leads to negative curvature, as illustrated in Figures 2 and 3, where the titration data have been fitted with reasonable success by the theoretical treatments in the presence[5] and absence[39] of salt, respectively.

Experimental evidence from investigations of other ionic polysacchardies is of interest in this respect. Muroga et al.[32] found significant negative curvature for titrations of (carboxymethyl)cellulose with degree of substitution (DS) of 1.55 carboxyl groups per monosaccharide. While the curvature was smaller than that predicted from the Poisson–Boltmann equation at low α, the decrease in curvature with increasing ionic strength agreed qualitatively with theoretical predictions. A similar curvature occurred for titrations in 0 and 0.2 M NaCl of (carboxymethyl)dextrans;[44] the curvature decreased as DS decreased, so that nearly linear plots resulted at DS = 0.35 above α = 0.3. Rinaudo and Milas[45] found nearly linear behavior in salt-free titration of a pectic acid sample of DS = 0.3. The titration behavior of pK' vs. α predicted by the nonlinearized equations is therefore broadly in agreement with experiment. Its use for extrapolation purposes seems justified, although the predicted curvature is not evident in our data and is opposite in sign to the apparent curvature observed at low ionic strength.

A further complication in the extrapolation is the possibility that the *N*-acetyl groups of the glucosamine residue become protonated at low pH. A study of dissociation constants of N-alkylated acetamides[46] indicated a typical pK of about 0.8. Occurrence of such protonation in our titrations would lead to apparent pK' (or pK'') values higher than if only carboxylic acid groups were present. The magnitude of this effect is shown in Figure 4 by the crosses, which represent the same points as the circles but corrected for protonation of *N*-acetyl groups (of total concentration equal to that of the carboxyl groups) with an assumed pK of 0.8. The correction is seen to be large enough to reduce significantly the apparent positive curvature at low α.

The theoretically predicted curvature becomes smaller as ionic strength increases, so that the extrapolation is less affected by uncertainty at higher ionic strengths. The best value of pK from cell A would thus appear to be that estimated above, pK = 2.86. Linear extrapolation of pK'' at 0.001 M NaCl leads to the somewhat higher values quoted in Table II averaging about $pK_0'' = 2.95$. The extrapolation in Figure 3 from the nonlinear equation gives about $pK_0'' = 2.85$, consistent with the results from cell A. The most probable value therefore appears to be about pK = 2.9 (±0.1), where the rather high error estimate reflects the uncertainties in extrapolation.

Mathews[47] arrived at pK values for hyaluronic acids from group A streptococcus and human umbilical cord of 2.93 and 3.04, respectively, on the basis of titrations in 0.1 M NaCl; the calculations of pK from the data involved compensating errors, however, since ionic strength effects on pK_0' were ignored and a smaller slope of pK' vs. α was assumed from theory than that found experimentally here. Laurent[11] used a different extrapolation procedure to ob-

tain pK = 3.21 at infinite ionic strength from his titration data in NaCl solution.

The estimation of pK for the D-glucuronic acid residue from titration data for the structurally similar chondroitin sulfates is also of interest in this respect. Chondroitin sulfate differs from hyaluronic acid only in that the *N*-acetylhexosamine residue is galactosamine rather than glucosamine and that a sulfate group, ionized in the pH range of the titrations, is substituted for the hydroxyl group at carbon 4 (in chondroitin 4-sulfate) of the galactosamine residue. The potentiometric titration data of Gilbert and Myers[48] gave linear plots of pK' vs. α for the neutralization of the carboxylic acid groups. The authors derived the values of pK_0' = 3.06 in 0.1 M NaCl and pK_0' = 2.92 in 1 M NaCl. Calculation of y_0 at I = 0.1 M with the appropriate ξ = 1.4 and a = 10 Å, as for hyaluronic acid, from the Stigter tabulations[5] yields y_0 = 1.8 at α = 1 and y_0 = 0.96 at α = 0, where ξ = 0.7 due to the residual ionized sulfate groups. The calculated change in pK' over this range is therefore 0.434 × 0.84 = 0.365 compared to the experimental value of 0.354.[48] The value of pK_0' at α = 0, corrected for the potential of the sulfate groups, would be pK_0' = 3.06 − 0.96 × 0.434 = 2.64. A similar calculation for 1 M NaCl gives a predicted pK' change from α = 0 to α = 1 of 0.16 (experimental value 0.10) and a corrected pK_0' of 2.76. These results agree within about 0.1 pK unit with the experimental (or expected) values (see Figure 1) for hyaluronic acid.

The value of pK derived for hyaluronic acid in this work is 0.3–0.4 pK unit lower than that of the monomeric analogue, D-glucuronic acid. This decrease may be due largely to the effect on pK of substitution in the polymer of the hexosamine residue for the hydroxyl group at carbon 4. Kohn and Kováč[29] found a decrease of about 0.3 unit in the pK of D-galacturonic acid when a methoxyl group was substituted at this position; substitution of three methoxyl groups at carbons 2, 3, and 4 led to decreases of 0.38 and 0.25 pK unit for D-galacturonic and D-glucuronic acids, respectively. The determination of the value of pK for the appropriate disaccharide of hyaluronic acid would be useful in testing whether substitution of the *N*-acetylhexosamine residue is actually responsible for the observed decrease of pK in the polymer.

Comparison to Electrophoretic Mobility. The electrophoretic mobility u_0 in the free-draining limit can be related to the electrostatic potential (or zeta potential ζ) at the hydrodynamic shear surface. For the case of a polyion like hyaluronate, presumably without a condensed ion layer, the hydrodynamic shear surface should lie at or near the polyion surface, where the latter may enclose strongly adsorbed solvent.

Schellman and Stigter[49] showed that u_0 for the uniformly charged infinite cylinder model could be written in terms of a reduced limiting mobility u_0'

$$u_0' = 6\eta e u_0/DkT = (e\zeta/kT)(1 + f_\perp)/2 \qquad (22)$$

where η is the viscosity of the solvent medium. The quantity $f_\perp$ is a correction factor for perpendicular flow, which is a function of x_0 and y_0 for the cylinder. For hyaluronate parameters $(1 + f_\perp)/2$ is very nearly unity, according to the tabulated values[49] over the ionic strength of interest.

Experimental mobility values must be extrapolated to C_2 = 0 in order to eliminate intermolecular effects on the flow. The only available data where such extrapolation has been performed appears to be that of Niedermeier and Gramling,[50] who observed the descending pattern at 1 °C in a Tiselius apparatus for a synovial fluid hyaluronate of high molecular weight (intrinsic velocity $[\eta]$ ca. 6000 cm^3 g^{-1}) in a Veronal buffer having I = 0.1 M and pH 8.6. The free-draining approximation can be expected to be valid for hyaluronate, as for most other polyions, although no data are available to test this assumption, which would require that mobility be independent of molecular weight. From the extrapolated results for four samples,[50] an average u_0 = 7 (±0.5) × 10^{-5} cm^2 V^{-1} s^{-1} can be derived, corresponding to u_0' = 1.0. Provided the ζ potential can be identified with the surface potential ψ_0, $u_0' = y_0$. The electrophoretic result is thus in agreement with our potentiometric value in Table II for 0.1 M salt, since 0.43/0.434 = 1.0. This agreement is of significance because it does not depend essentially on the physical model used. Further experimental values of u_0 as a function of α and C_3* would be desirable in verifying this admittedly isolated result. The identification of ζ and surface potentials, if valid, probably holds only for polyions of low charge density. Stigter has shown[51] that for polymethacrylates and DNA, the electrophoretic (or conductometric) charge is, in general, smaller than that derived from titration, i.e., $e\zeta/kT < y_0$ at larger ξ.

In summary, our results provide evidence that the form of titration data for a polyion of $\xi < 1$ are described well by the uniformly charged cylinder model with respect to change of ionic strength, although the magnitude of the potential appears to be overestimated for realistic geometric parameters.

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Appendix

The interpretation of cell potentials in this work required attention to the effect of the polyion on the activities of small ions. The activity of NaY (component 3) in solutions of partially neutralized hyaluronic acid (component 2) may be written in the usual way in terms of the activities a_i, molalities m_i, and activity coefficients γ_i of the univalent ionic species i

$$a_3 \equiv a_{Na}a_Y = m_{Na}m_Y\gamma_{Na}\gamma_Y = m_3^2(1 + \nu X/\alpha)\gamma_\pm^2 \qquad (A1)$$

where eq 3 (and the relations following it) and eq 7 have been used and where the mean ionic activity coefficient $\gamma_\pm$ has its usual definition $\gamma_\pm^2 \equiv \gamma_{Na}\gamma_Y$. For the case of dialysis equilibrium, with equilibrium solvent values denoted by an asterisk, $a_3 = a_3^* = m_3^{*2}\gamma_\pm^2$ so that

$$\gamma_\pm^2/\gamma_\pm^{*2} = m_3^{*2}/[m_3^2(1 + X)] \qquad (A2)$$

for the fully neutralized polymer acid: ν = 1, α = 1. Replacement of m_3^*/m_3 (with an error of the order of the volume fractions of solutes) by $C_3^*/C_3 = 1 - A_1X$ (from solving eq 1-1 with A_2 = 0) leads to

$$\gamma_\pm^2 = \gamma_\pm^{*2}(1 - A_1X)^2/(1 + X) \qquad (A3)$$

$$\gamma_\pm^2 = \gamma_\pm^{*2}(1 + \phi_pX)/(1 + X) \qquad (A_1X \ll 1) \qquad (A4)$$

where the A_i are virial coefficients for salt distribution. Equation A4 results from the substitution of the relation $-A_1 = \phi_p/2$ of the line charge theory,[3] where ϕ_p is the osmotic coefficient. If, in addition, $\gamma_\pm^*$ is set equal to unity, the additivity rule (eq 55 of ref 3) of the line charge theory is recovered.

From dialysis equilibrium measurements Preston et al.[8] obtained experimental values of $\gamma_\pm$ at various m_3^* below 0.025 m for NaCl in solutions containing fully neutralized sodium hyaluronate. Equation A3 with theoretical A_1 values calculated[1] with ξ = 0.70 from the rigid-cylinder or line charge models does not reproduce their results well, especially at high A_1X. Preston et al. fitted their data with

a modification of eq A4 suggested by Katchalsky et al.,[52] in which $\gamma_\pm$* was replaced by $\gamma_\pm'$, which was regarded as a mean activity coefficient for the "free" ions in the polymer solution. The ratio $\gamma_\pm/\gamma_\pm'$ and ϕ_p were then treated as adjustable constants to fit the data. As recognized by these authors,[8,52] experimental data may often be fitted equally well by eq A4; we find a good fit with $\phi_p = 0.75$ as the only variable parameter, even for large A_1X, where eq A4 must be regarded as empirical.

The problem encountered in potentiometric titration is the estimation of activity coefficients in solutions where m_2 and m_3 are known but m_3* is not. For this purpose the form used by Preston et al. is convenient with the "free" ion coefficient $\gamma_\pm'$ taken as the mean activity coefficient at m_3 in the polymer-free solution. All of the experimental values of $\gamma_\pm$ can then be fitted within 1% with $\phi_p' = 0.73$; where ϕ_p' is the value of ϕ_p evaluated in this way. This empirical form reduces to eq A4 as X approaches zero, since $\gamma_\pm'$ then approaches $\gamma_\pm$*.

An alternative additivity rule for the activity coefficient γ_c of the counterion c in a solution containing excess salt at concentration m_3 has been proposed[53]

$$\gamma_c = \frac{\gamma_c' + \gamma_p X}{1 + X} \tag{A5}$$

where γ_c' is the activity coefficient of c in the polymer-free solution at m_3 and γ_p is the corresponding activity coefficient in the salt-free solution. When, in addition, the activity coefficient of the coion Y is approximated by its value γ_Y' in the polymer-free solution at m_3, as suggested by Oosawa,[54] the mean activity coefficient $\gamma_\pm$ becomes

$$\gamma_\pm^2 = (\gamma_c' + \gamma_p X)\gamma_Y'/(1 + X) = \gamma_\pm'^2(1 + \gamma_p X/\gamma_c')/(1 + X) \tag{A6}$$

Equation A6 has enjoyed considerable success an an empirical rule for fitting data; its resemblance in form to eq A4 is evident. It is of interest for our purposes to test eq A6, as well, with the data of Preston et al. When the individual ionic activity coefficients are taken according to the Bates–Guggenheim convention[21] for NaCl, $\gamma_{Na}' = \gamma_{Cl}' = \gamma_\pm'$ at m_3, and γ_p is taken from the line charge theory[3] for the salt-free case, $\gamma_p = \exp(-\xi/2) = 0.705$ for $\xi = 0.70$, values of $\gamma_\pm$ calculated from eq A6 agree with the experimental values within 2% throughout. Somewhat higher experimental values of γ_p, based on potentiometric measurements on salt-free solutions of sodium hyaluronate with a sodium ion sensitive glass|test solution|calomel cell, have been reported.[8,9] These values, which range from 0.81 at $C_2 = 0.002$ M to 0.72 at $C_2 = 0.010$ M, are subject to some uncertainty because of errors due to liquid-junction potential, as discussed in the text.

References and Notes

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