Physical Properties of Cartilage Proteoglycans†

Clifford B. Woodward,‡ John Hranisavljevic,§ and E. A. Davidson*

ABSTRACT: A detailed study of the molecular weight of several preparations of cartilage proteoglycan was performed by sedimentation equilibrium methods. Weitht average molecular weights range from 37,000 upward and display considerable heterogeneity. The chondroitin 4-sulfate chains are nearly homogeneous and have weight-average molecular weights of 13,000. The heterogeneity most likely arises from variations in the polypeptide core due to differing levels of saccharide substitution or catheptic proteolysis. Evaluation of the data suggests that aggregation may occur as well although a clearly defined stoichiometry is not present. A comparison to molecular weight data derived from sedimentation velocity and viscosity measurements is offered. The derived polysaccharide chains although not significantly polydisperse may associate to some degree.

There have been a number of studies reported in the literature on the molecular weights and shapes of proteoglycans and chondroitin sulfates (Blix and Snellman, 1945; Mathews and Dorfman, 1953; Mathews, 1956; Mathews and Lozaityte, 1958; Malawista and Schubert, 1958; Gerber *et al.*, 1960; Partridge *et al.*, 1961; Tanford *et al.*, 1964; Marler and Davidson, 1965; Muir and Jacobs, 1967; Luscombe and Phelps, 1967; Sajdera and Hascall, 1969; Dunstone and Franek, 1969). The values reported for the molecular weights range from 10^5 to 8×10^6 for the protein–polysaccharides and from 1.3×10^4 to 6×10^4 for the chondroitin sulfates. The higher values for the intact complex invariably come from techniques which do not measure the molecular weight directly, but rather rely on various mathematical models which are based on assumptions that may not apply to the system in question. We have examined preparations made several years ago by the procedure of Malawista and Schubert (1958) and Gerber *et al.* (1960) as well as several preparations of our own, using the thermodynamically sound method of sedimentation equilibrium. The use of relatively low ionic strength as employed in many of the studies listed above, was avoided because molecular weight studies of highly charged polyions by viscometric and sedimentation velocity methods should be performed in the presence of sufficient supporting electrolytes so that charge–charge interactions are minimized. This study will provide a strong basis for caution when use of low ionic strength media is contemplated for the determination of the molecular weights of highly charged polyions *via* sedimentation velocity and viscometry.

Theory

Sedimentation Equilibrium. In the equation for the equilibrium distribution of a macromolecular solute (component 2 by the numbering system of Scatchard, 1946) in the centrifugal field there are several assumptions related to the ideal behavior of component 2. It is assumed that $\bar{v}_2$ does not change

$$\frac{d \ln c_2}{dr^2} = M_2(1 - \bar{v}_2\rho_x)\frac{(\omega^2)}{2RT} \quad (1)$$

with concentration or pressure and that ρ_x, the density of the complete solution, does not change with radial position. In actual fact, ρ_x, always changes in H_2O–salt–macromolecule systems because the salt sediments slightly and the macromolecules greatly; this is usually not important, but may become significant at high salt or macromolecule concentrations. The value of $\bar{v}_2$ may be the limiting factor in the accuracy of an experiment and should be experimentally evaluated.

The following discussion is derived in part from studies published by Roark and Yphantis (1969) and the reader is referred to that manuscript for complete details of the theory.

If there are only two ideal sedimenting species present in the system, an equation may be written for the purposes of obtaining the molecular weights of both species (Roark and Yphantis, 1969). This equation is valid whether or not the two species are involved in a chemical or physical equilibrium with each other

$$M_w(r) = -M_1M_2[1/M_n(r)] + M_1 + M_2 \quad (2)$$

where M_1 and M_2 are the two sedimenting species. Thus a graph of $M_w(r)$ *vs.* $1/M_n(r)$ should yield a straight line whose slope is $-M_1M_2$ and whose intercept is $M_1 + M_2$. In general, Roark and Yphantis (1969) have shown

$$M_k(r) = M_1M_2[1/M_{k-1}(r)] + M_1 + M_2 \quad (3)$$

where $M_k = M_n$, M_w, M_z, and M_{z+1} as $k = 0$, 1, 2, and 3, respectively. If there are more than two species present (*e.g.*, nonstoichiometric aggregation), such a plot may tend to curve upward; if there is nonideality present, a two-species plot will tend to curve downward

In the latter case, the determination of M_1 may not be possible, especially if the downward curvature is great. Several equations have been developed for the evaluation of such data. One of these is

$$2M_z - M_{z+1} = -M_1M_2(2/M_w - 1/M_z) + (M_1 + M_2) \quad (4)$$

† From the Department of Biological Chemistry, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received September 20, 1971.* Supported by U. S. Public Health Service Grant AM12074.

‡ Present address: Biochemisch Laboratorium Der Rijksuniversiteit, Utrecht, The Netherlands.

§ Present address: Department of Chemistry, Novi Sad University, Novi Sad, Yugoslavia.

* To whom all correspondence should be addressed.

A plot of $(2M_z - M_{z+1})$ *vs.* $(2/M_w - 1/M_z)$ should give an essentially straight line if only two species are present.

In the case of a nonideal solute which undergoes a specific reversible association ($NM_1 \rightarrow (M_1)_n$), the molecular weight averages may be written in the form of virial equations, in which most of the nonideality is confined to the second virial coefficient

$$\frac{1}{M_{w,app}} = \frac{1}{M_{w,id}} + 2B_1c = \frac{1}{M_1} + 2(B + B_1)_c + 3(c^2 + 4D_c^3 + \ldots \quad (5)$$

$$\frac{1}{M_{n,app}} = \frac{1}{M_{n,id}} + B_1c = \frac{1}{M_1} + (B + B_1)_c + Cc^2 + Dc^3 + \ldots \quad (6)$$

Molecular weight moments[1] can be derived from these expressions which are free of the second virial coefficient; one such moment, M_{Y8} is also free of the third and fourth virial coefficients and is useful for the estimation of monomer molecular weights.

If the sedimenting system contains three ideal species with moments σ_1, $\sigma_2 = N\sigma_1$, and $\sigma_3 = M\sigma_1$ and with $\sigma_1 < \sigma_2 < \sigma_3$, equations may be derived which are analogous to those for an ideal two-species system

$$I_2 = -\sigma_2\sigma_3\left(\frac{1}{I_1}\right) + \sigma_2 + \sigma_3 \quad (7)$$

$$I_3 = -\sigma_2\sigma_3\left(\frac{1}{I_2}\right) + \sigma_2 + \sigma_3 \quad (8)$$

where I_1, I_2, and I_3 are values called "intermediates." Such plots of I_2 *vs.* $1/I$ and of I_3 *vs.* $1/I_2$ are called "three-species" plots and give a straight line when only three ideal species are present.[2] All of the moments and intermediates were calculated with the aid of computer programs developed by Roark and Yphantis (1969)

Viscosity. Tanford's (1955) equation was employed to evaluate the relationship between intrinsic viscosity and intrinsic kinematic viscosity

$$[\eta_x] = [\nu_x] + \frac{1 - \bar{\nu}_x\rho_0}{100\rho_0} \quad (9)$$

where x is a pure component; $[\nu_x]$ is the intrinsic kinematic viscosity and the value actually measured; $\bar{\nu}_x$ is the partial specific volume of x, and ρ_0 is the density of the solvent. The intrinsic viscosity was measured for the purpose of using several of the available theories concerning the size and shape of macromolecules. The Scheraga–Mandelkern (1953) theory, for example, uses the concept of an effective hydrodynamic ellipsoid. Their expression

$$\beta = \frac{N[\eta_x]^{1/3}\eta_0[s]_x}{M_x^{2/3}(1 - \bar{\nu}_x\rho_0)} \quad (10)$$

has been frequently used for molecular weight determinations on the various proteoglycans. Measurements are usually made of $[\eta_x]$, $[s_x]$, η_0, $\bar{\nu}_x$, and ρ_0 and a value of β assumed that is somewhere between 2.12 and 2.15. This enables calculation of a molecular weight which is not too dependent upon the value of β chosen but which may not enable one to distinguish between prolate and oblate ellipsoids at values of β between 2.12 and 2.15.

Sedimentation Velocity. Sedimentation constants, s_x, at a particular solute (x) concentration were measured by following the migration of the peak maximum. The migration rate at zero experimental time was taken to be the value desired at a particular solute concentration. The data for several solute concentrations were extrapolated to zero concentration by plotting $1/s_x$ *vs.* c and the value of s_x^0 or $[s_x]$ obtained.

Partial Specific Volume. These were determined according to the criteria of Cassasa and Eisenberg (1960, 1961) and were taken to be

$$\lim_{m_2 \to 0}\left(\frac{\delta v}{\delta g_2}\right)_{T,P,g_{i\neq 2}} \quad (11)$$

at dialysis equilibrium where V is the volume of the solution, g_i is the grams of i, and the components are numbered according to Scatchard (1946). Values were extrapolated to zero concentration and for comparison purposes, corrected for the counterion present. The optical rotatory dispersion (ORD) behavior of the proteoglycan is substantially the same at high ionic strength regardless of the nature of the counterion (Na^+, Ca^{2+}, Ba^{2+}, Sr^{2+}, and K^+) suggesting that gross changes in shape do not occur. The partial specific volumes were experimentally evaluated for the sodium form only.

Experimental Section

Ultracentrifuge. A Beckman Model E analytical ultracentrifuge was used throughout. Rayleigh interference optics were used for all equilibrium sedimentation experiments and schlieren optics were used for all sedimentation velocity experiments. The optics were aligned with the light source focused on the two-thirds cell plane for sapphire windows and 12-mm path-length cells. The precautions mentioned by Ansevin *et al.* (1970) for sedimentation equilibrium experiments were followed. A polarizer was placed over the light source with its plane of polarization parallel to the long axis of the slit; the push-pull slit assembly was not employed; polyvinyl chloride window cushions were used in the centrifuge cells and were run with a water blank overnight to eliminate any significant differences between before and after blanks. All experiments were carried out at 25°. Spectroscopic type II-G or 103a plates (Eastman Kodak, Inc.) were used and were developed with Kodak HPR developer. A Nikon microcomparator was used for all plate analysis and equilibrium sedimentation data were analyzed as described.

The sedimentation velocity studies were run at 56,000 rpm and at 25°. A 12-mm path length, 4° single-sector cell was employed and was always filled with the same volume of solution, thus ensuring that all extrapolations to zero-time migration rate were made at the same radius. Metallographic plates (Eastman Kodak, Inc.) were used and were developed in Kodak, D-11 developer.

Viscometry. Solutes, as their sodium salt, were made up in the appropriate solvent at the highest solute concentrations to be tested, and serial dilutions with solvent were made

[1] The apparent reduced molecular weights are as defined by Yphantis (1964)

$$\sigma_i(r) = M_{i,app}\frac{(r)(1 - \bar{\nu}\rho)\omega^2}{RT}$$

where $\bar{\nu}$, ρ, ω, R, and T have their usual meanings.

[2] Dr. Roark, personal communication.

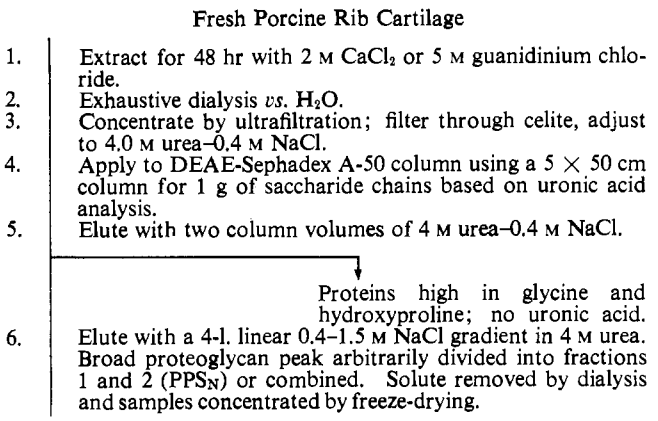

FIGURE 1: Flow chart for proteoglycan preparation. All steps subsequent to extraction are carried out at 4°. Material eluted in step 5 resembles collagen in amino acid composition; detailed characterization of this fraction and its relationship to the crude extract will be described subsequently.[1]

to obtain lower concentrations Analysis for uronic acid was done using the orcinol method (Mejbaum, 1939). Duplicate analyses were run and, using the weight fraction of polysaccharide from amino acid analysis, the amount of protein–polysaccharide was calculated. All solutions were filtered through 0.45 μ Millipore filters prior to use and measured values were corrected for loss of material.

The viscometers used were of a modified Ostwald type with a flow time for water of about 220 sec at 25°. The viscometers were cleaned with concentrated H_2SO_4–$K_2Cr_2O_7$, rinsed exhaustively with water, and dried between individual runs. Temperature was maintained at 25.00 ± 0.003° and 10–15 readings were taken per sample for averaging.

Partial Specific Volume. Solutes, as their sodium salt, were made up in the solvent to be used and dialyzed to equilibrium. The measurements were carried out on a Cahn-RG electronic microbalance with a modified 2700 density cell attachment. Data were obtained at several different concentrations by dilution with dialysate. The temperature was maintained at 25.0°. Concentrations were determined from the sodium salt on a dry weight basis and corrected from sodium to hydrogen after determination of the sodium content by atomic absorption analysis.

Sample Preparation. The samples studied were prepared by a modification of the Sajdera–Hascall (1969) method using 2 M $CaCl_2$ or 5 M guanidinium chloride as extractant. One set of experiments were carried out using 2 M $CaCl_2$ without any regulation of the pH during the course of the extraction. Within 20 min the pH dropped to 5.2 and eventually went below pH 5. In a second series, the pH was maintained between 6.5 and 7.0 throughout the $CaCl_2$ extraction by periodic additions of alkali. The other details of the extractions were substantially the same and are summarized in the flow diagram presented in Figure 1. The protein–polysaccharides isolated in the first $CaCl_2$ experiment are designated PPS_H and those isolated in the second experiment are designated PPS_N.

Saccharide chains and peptides were resolved from the various fractions by treating the intact protein–polysaccharides at pH 11.8 for 8 hr at 0–4° in the presence of freshly prepared 0.2 M sodium sulfite. The mixture of peptide and polysaccharides was resolved on a Dowex 1 acetate form column by elution with a stepwise linear NaCl gradient. The free peptide emerged in the first gradient and the polysaccharides in the second. The seryl residues which function as attachment points for polysaccharide chains on the peptide were converted to cysteic acid under the conditions described (Simpson *et al.*, 1971).

TABLE I: Analyses of Proteoglycan Fractions.[a]

	Uronic Acid	Glucosamine	Galactosamine	Sulfate	Amino Acid
PPL	26.3	1.6	24.8	9.60	9.7
PPS_H	27.2	1.4	25.3	9.5	8.9
PPS_{N1}	25.4	1.8	26.2	8.7	10.1
PPS_{N2}	26.7	1.9	26.0	9.0	9.7
PPS_{gu}	27.1	1.7	25.8	8.9	9.4
C-4S (commercial)	41.7	0.1	40.6	16.1	0.2
C-4S (from PPS_{N1})	40.9	0.2	41.0	15.8	0.1

[a] Analytical data for proteoglycan and polysaccharide preparations. Figures are per cent by weight. Uronic acid analysis was by the method of Bitter and Muir (1962) and sulfate by the method of Lloyd (1959). Amino acids and amino sugars were quantitated on an automatic amino acid analyzer following hydrolysis with 6 N HCl *in vacuo* for 24 hr. Calculations were performed utilizing a computer program developed in this laboratory. Additional details regarding amino acid composition and fractionation properties will be reported separately. J. Hranisavljevic and E. A. Davidson, 1971, in preparation.

Results

Sedimentation Equilibrium. Sedimentation equilibrium studies were carried out on the proteoglycan fractions as well as on a commercial sample of chondroitin 4-sulfate (Na salt) (Super Special Grade, lot 4200, Seikagaku Kogyo Co. Ltd., Tokyo), chondroitin 4-sulfate chains isolated from the proteoglycan preparations and a proteoglycan sample originally isolated in 1966 by the procedure of Gerber *et al.* (1960) and kept in a desiccator over silica gel in the interim (PPL). When the latter sample was examined in 1966, it appeared quite heterogeneous but no conclusions regarding association behavior could be drawn. Analytical data for all fractions are presented in Table I. With the development of the "two-species" plot and its associated calculations new avenues of data analysis became available to us.

The figures which follow are representative and show the powers and limitations of the analytical techniques. Figure 2 shows some of the molecular weight moments of PPL in 4.50 M NaCl; the molecular weights, M_k, may be calculated using the formula given in the figure legend. It can be seen that all the moments extrapolate back to the same molecular weight of 21,500 ± 500. When these data are examined on a two-species plot (Figure 3), they are found to lie on a straight line which has a slope between that for monomer–dimer and monomer–trimer systems, indicating the presence of higher aggregates. The system may obey a monomer–dimer relation at the lowest concentrations but is almost certainly polydisperse and may well contain aggregates of undefined stoi-

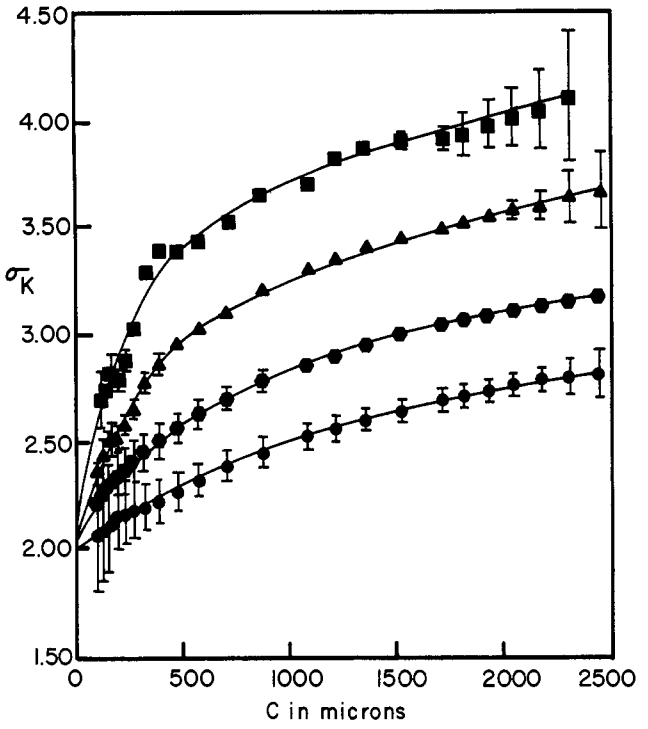

FIGURE 2: Molecular weight moments for PPL in 4.50 M NaCl. rpm = 26,230; $M_k = (\sigma_k)$ (10,480); loading concentration = 0.8 g/l. (●) σ_{yl}, (⬬) σ_n, (▲) σ_w, and (■) σ_z.

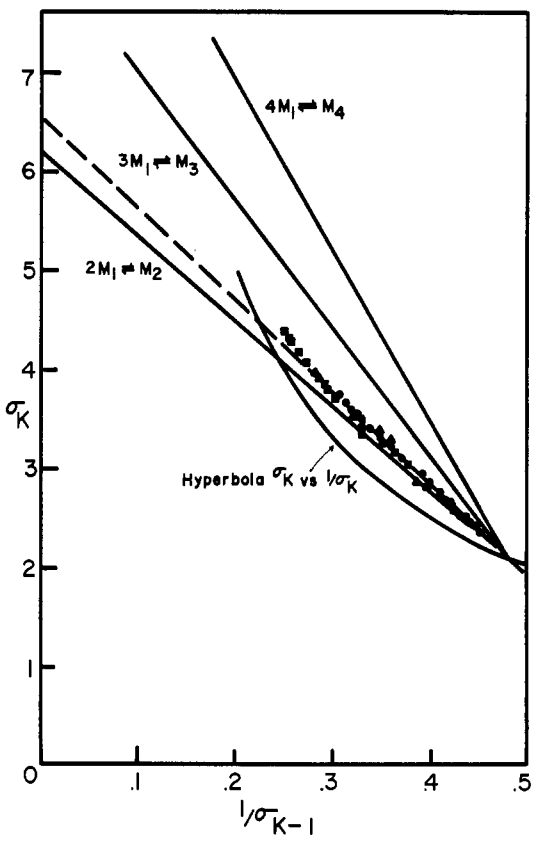

FIGURE 3: Two species plot of data illustrated in Figure 2. (●) σ_w *vs.* $1/\sigma_n$, (▲) σ_z *vs.* $1/\sigma_w$, and (■) σ_{z+1} *vs.* $1/\sigma_z$.

chiometry. In addition the extrapolated value of 21,500 may represent a minimum size and not a monomer in equilibrium with larger aggregates.

Figure 4 shows the behavior of PPS_H fraction 1. A second fraction isolated by chromatography on DEAE-Sephadex A-50 had essentially the same properties with a minimal molecular weight about 7000 higher than that of fraction 1. The minimal molecular weight for fraction 1 based on extrapolation to zero concentrations, was 37,000 ± 500. Both the equilibrium data (σ *vs.* C) as well as the two species plots indicate substantial heterogeneity in the sample. It seems reasonable to ascribe at least a portion of this heterogeneity to catheptic proteolysis occurring during the extraction procedure. This conclusion is substantiated by the fact that the molecular weights of the PPS_N fractions are somewhat higher than those of PPS_H fractions. The minimal or monomer molecular weight of PPS_N fraction 1 was 60,200 ± 2000 and that of fraction 2 was 140,000 ± 5000. The two fractions seem to be related in that PPS_N fraction 1 has an upper molecular weight, M_w, of around 140,000 and PPS_N fraction 2 ranged upward from 140,000. Figure 5 illustrates the molecular weight parameter σ_w for a proteoglycan sample prepared by guanidinium chloride extraction. Heterogeneity is still evident and is also reflected in the nonlinearity of the two species plot (Figure 6). Although some association stoichiometry may exist at lower concentrations, higher aggregates of undefined composition are apparently present as well. The extrapolated minimal molecular weight is the same as that of PPS_N fraction 1 within experimental error.

Presumptive nonideality of solute behavior was observed in several cases when the solvent was 6 M guanidine hydrochloride. In Figure 7 are plotted some of the molecular weight moments of PPS_N fraction 1. Note in particular, σ_w and σ_z, which both tail off toward higher concentrations. For a purely ideal associating system, these two σ values would continue

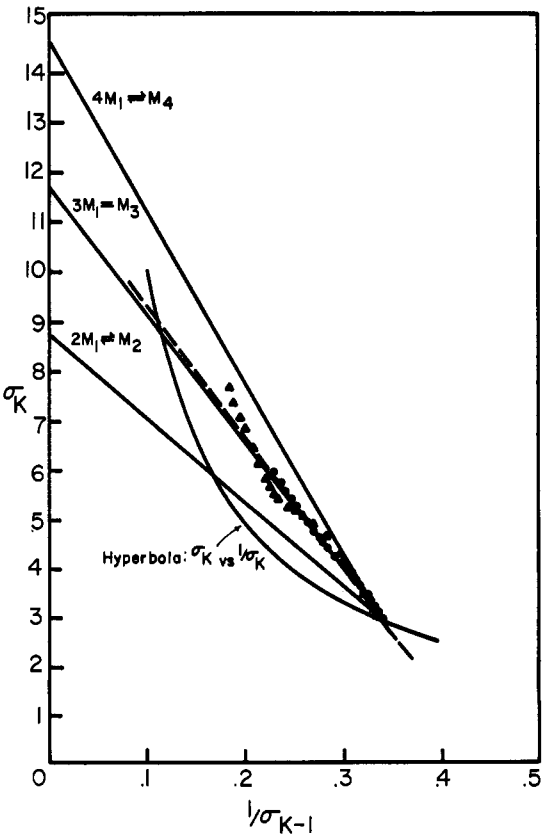

FIGURE 4: Two-species plot of molecular weight moments of PPS_H in 8 M urea–1.50 M NaCl. rpm = 24,125; $M_k = (\sigma_k)$ (12,772) (●) σ_w *vs.* $1/\sigma_n$ and (▲) σ_z *vs.* $1/\sigma_w$.

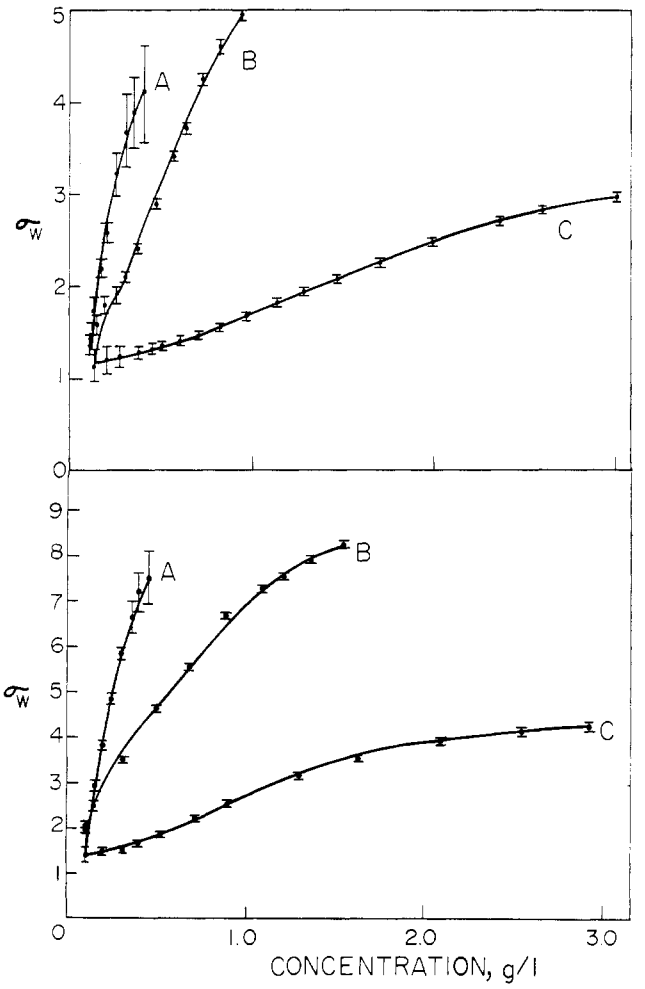

FIGURE 5: Molecular weight parameter σ_w for cartilage proteoglycan prepared by extraction with 5 M guanidinium hydrochloride. Concentration units are mg/ml; solvent is 2.0 M KCl. Upper curves are data at 7200 rpm and lower curves are data at 15,000 rpm. A, B, and C refer to initial loading concentrations of 1.6, 0.8, and 0.4 g per l., respectively.

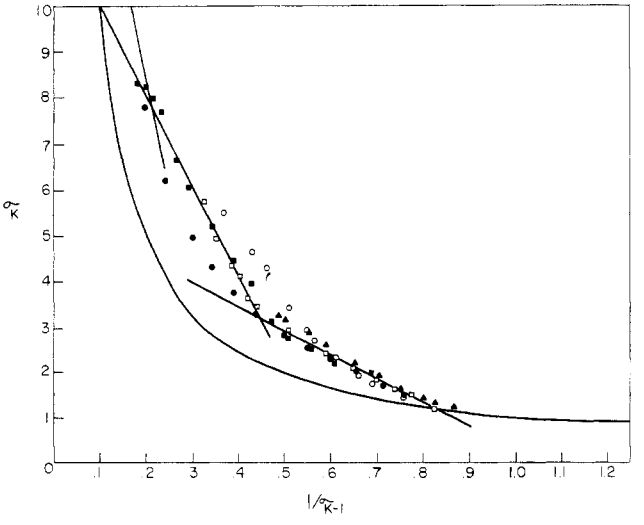

FIGURE 6: Two-species plot for data illustrated in Figure 8; upper set. (▲) 0.4 gl/., σ_w *vs.* $1/\sigma_n$; (□) 0.4 g/l., σ_z *vs.* σ_w; (●) 0.4 g/l., σ_{z+1} *vs.* $1/\sigma_z$; (○) 0.8 g/l., σ_w *vs.* $1/\sigma_n$; (■) 0.8 g/l., σ_z *vs.* $1/\sigma_w$. Linear segments indicate possible stoichiometric fits but the extent of the curvature present makes it unlikely that specific polymers are present in significant concentrations.

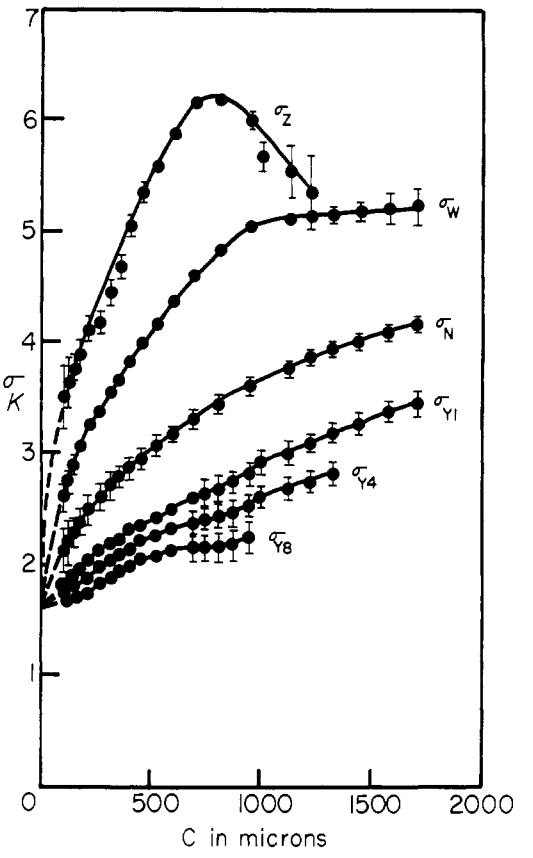

FIGURE 7: Molecular weight moments of PPS_N fraction 1 in 6 M guanidinium chloride. rpm = 14.360; $M_k = (\sigma_k)$ (22,640); loading concentration = 1.0 g/l.

to increase smoothly as the various σ_y values do. When a two-species plot (Figure 8) is made of these data, the upper part of the plot starts to fall rapidly giving an initial indication of nonideality.

A similar plot using derived moments is shown in Figure 9. In this case, both heterogeneity and associative behavior are indicated. There is no clear stoichiometry of association although species weighing three or more time that of the monomer are apparently present.

The two-species plot for a sample of commercial C-4S (Figure 10) examined in 1.5 M NaCl–8 M urea exhibits monomer–trimer or monomer–dimer–oligomer properties at lower concentrations. Identical results were obtained with C-4S chains isolated from either PPS_H or PPS_N. Since these samples are relatively homogeneous, some association is apparently taking place. The high charge density of the chains (one negative charge per monosaccharide unit) might argue against this but molecular models indicate that one surface of the chains is relatively uncharged, exhibiting axial hydrogens only, and may be a locus for association. A more detailed study of this interaction is currently in progress.

All of the experiments reported here were repeated at least three times with the same results. The C-4S chains were also run at low Γ (0.1 M NaCl) and gave essentially the same data. There was a small but significant increase in M_w at the lower Γ which amounted to approximately 5% of the M_w at higher Γ.

Sedimentation Velocity. Intrinsic Svedberg coefficients were determined on PPS_N fraction 2 in 0.05 M NaCl–0.01 M Hepes buffer (pH 6.5) and in 4.50 M NaCl–0.1 M Hepes buffer

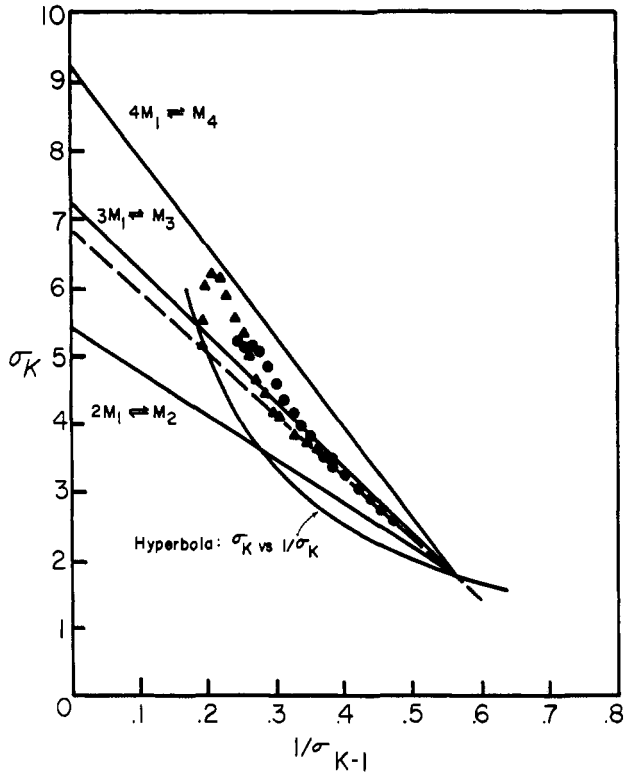

FIGURE 8: Two species plot of data illustrated in Figure 5. (●) σ_w *vs.* $1/\sigma_n$ and (▲) σ_z *vs.* $1/\sigma_w$.

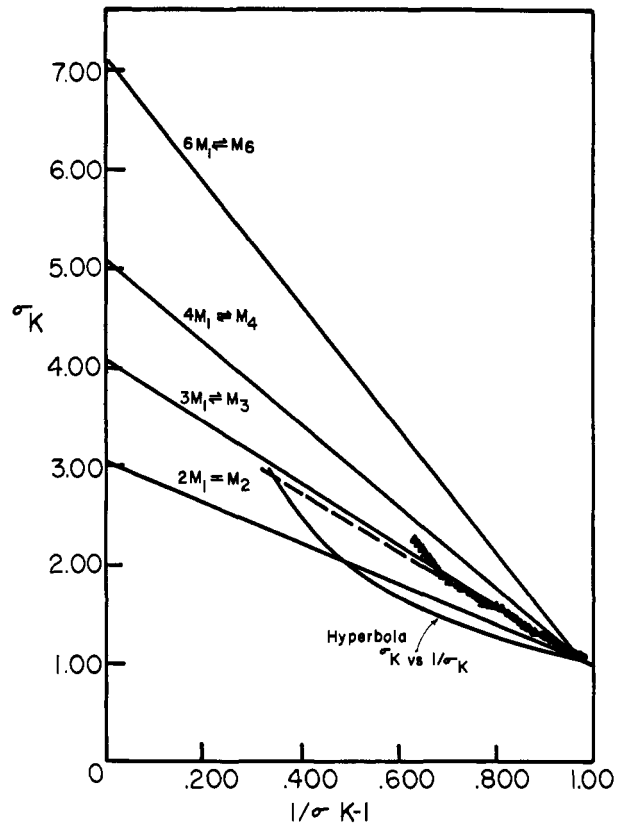

FIGURE 10: Two species plot of molecular weight moments of commerical chondroitin 3-sulfate chains. rpm = 22,315; $M_k = (\sigma_k)$ (12,963); loading concentration = 0.5 g/l. (●) σ_w *vs.* $1/\sigma_n$ and (▲) σ_z *vs.* $1/\sigma_w$.

(pH 6.5). At the low Γ the sedimentation coefficient was very concentration dependent (see Figure 11). The data at high Γ were not as concentration dependent and extrapolated to an intrinsic Svedberg coefficient of 5.1.

Partial Specific Volume. The apparent partial specific volume of PPS_N fraction 2 in 0.05 M NaCl–0.01 M Hepes buffer (pH 6.5) was measured as a function of concentration and extrapolated to a value of 0.591 at infinite dilution (Figure 12).

Viscosity. The viscosities of the various protein–polysaccharide complexes are rather high compared to ordinary globular proteins and have a marked dependence on concen-

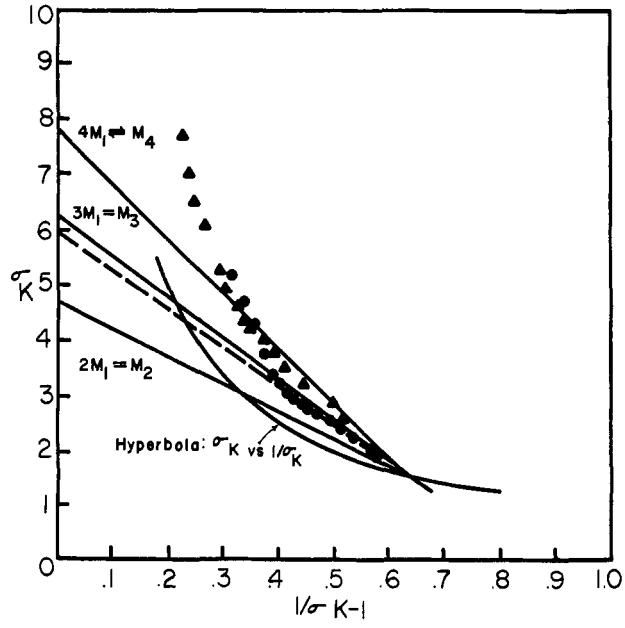

FIGURE 9: Two-species plot of ideal molecular weight moments derived from data presented in Figure 5. (●) σ_{y2} *vs.* $1/\sigma_{y1}$ and (▲) σ_{y3} *vs.* $1/\sigma_{y2}$.

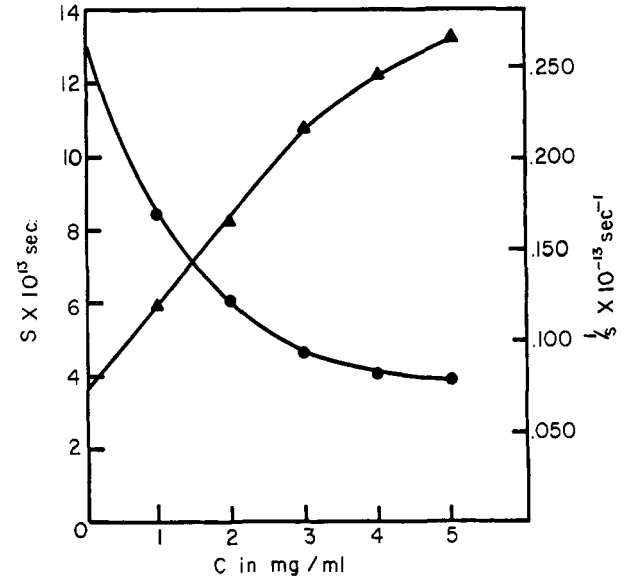

FIGURE 11: Sedimentation coefficient, *s*, and $1/s$ *vs.* *C* plots for PPS_N fraction 2 in 0.05 M NaCl–0.01 M Hepes buffer (pH 6.5). (●) = *s vs. C*; ▲ = $1/s$ *vs. C*; s_0 = 13.3 from *s vs. C*, 14.3 from $1/s$ *vs. C*. At $\Gamma = 4.50$, $s_0 = 5.1$.

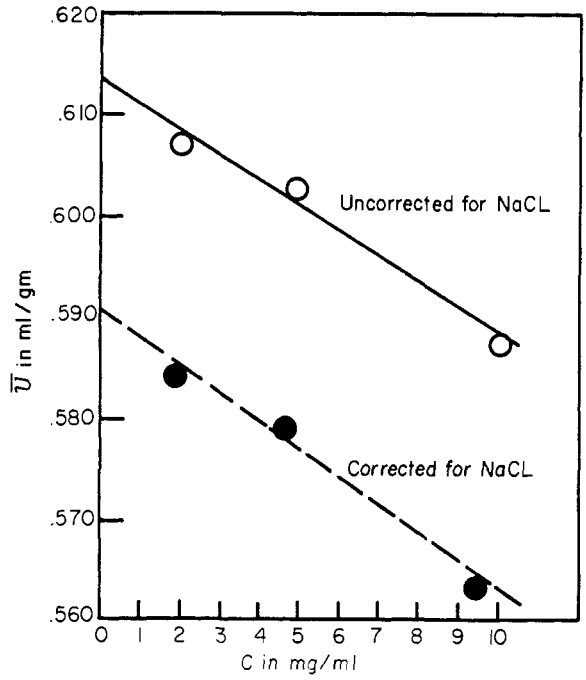

FIGURE 12: Partial specific volume determination on PPS_N.

tration and ionic strength. The specific viscosities of the cartilage proteoglycan at high ionic strength are presented in Figure 13. The large positive slope of the viscosity suggests that association is occurring leading to a shape (axial ratio) change. The viscosity of the PPS_N fractions in 1.5 M $CaCl_2$ is exactly the same as that for PPS_H in the same solvent, although the monomer molecular weights of these

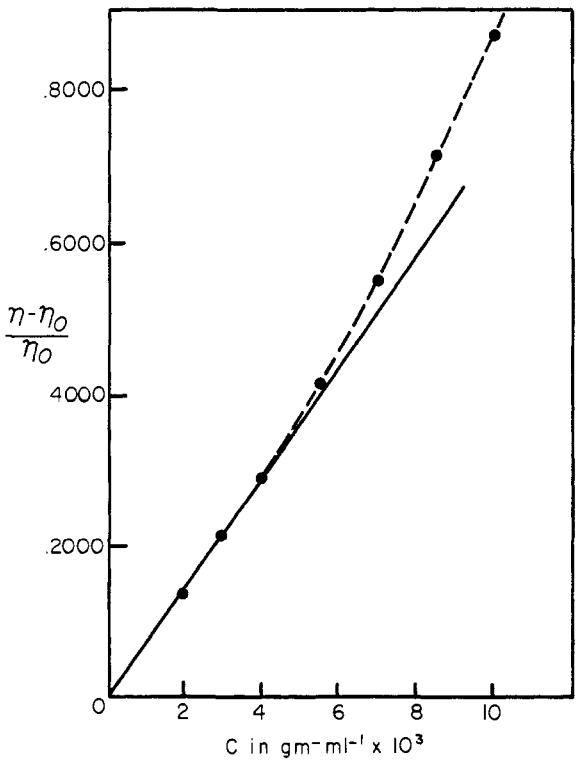

FIGURE 13: Specific viscosity of PPS_H in 1.50 M $CaCl_2$ (pH 6.5).

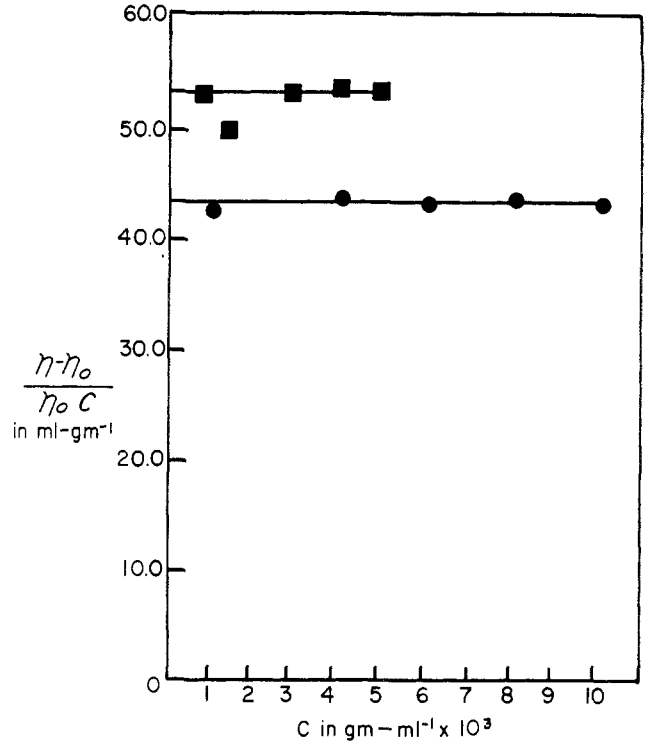

FIGURE 14: Reduced viscosity of chondroitin 4-sulfate chains in 1.50 M $CaCl_2$, pH 6.5 (●), or 0.05 M NaCl, pH 6.5 (■). Chains were isolated from PPS_N fraction 1 as described.

species are different. This again suggests that aggregation is occurring to produce the observed viscosity patterns.

The viscosity of chondroitin 4-sulfate was investigated in various solvents. The reduced viscosities of several preparations in different salts at $\Gamma = 4.5$ are the same, and all such plots had nearly zero slope. The reduced viscosity at $\Gamma = 0.05$ extrapolated to a different $[\eta]$ but also had zero slope. A slight change in shape is indicated by the difference in $[\eta]$ at high and low Γ's but aggregation is also a possibility (Figure 14).

In order to get a qualitative picture of the shape of these molecules, several viscosity theories were applied to our data (see Table II). At the lower ionic strength, the value of β in the Scheraga–Mandelkern theory gives an axial ratio which appears to be totally unrealistic. There are several possible causes for this: the presence of a large primary charge effect, the presence of an aggregating system in which the "polymers" are much more asymmetric than the monomers, or the presence of a large electroviscous effect in the viscometer. By doing the experiments at high Γ, the primary charge effect is largely eliminated. If this were the only cause of the concentration dependence of s at low Γ, the value of s at high solute concentrations and high Γ should increase to match those at low solute concentrations and low Γ. This is not the case in our system. The electroviscous effect is generally not large enough to alter the observed $[\eta]$ to a value low enough to correct β to a reasonable value (Yang, 1961). Aggregation to a much more asymmetric particle seems most probable but would have to be extreme to cause a large enough change in s upon dilution of solute at low Γ (Schachman, 1959). It seems likely that the discrepancies are in part due to a combination of the above effects and to the fact that the Scheraga–Mandelkern equation is not applicable to the data at low Γ because the molecules do not conform to the rigid, nonsolvated, ellipsoid upon which the theory is based.

TABLE II: Evaluation of Viscosity Results.[a]

Equation	Ionic Strength	Assumed	Calculated	Known
S	0.06	$M = 2 \times 10^5$	$\beta = 8.3 \times 10^6$ $a/b = \gg 300$	$s_0 = 14.3 \times 10^{-13}$ $[\eta] = 2.5$; $\eta_0 = 1.01 \times 10^{-2}$
S	0.06	$M = 8 \times 10^5$	$\beta = 3.3 \times 10^6$ $a/b = 150$	$\rho = 1.00$; $\bar{\nu} = 0.59$ $M = 2 \times 10^5$ (PPS_N fraction 2)
S	4.5	$M = 2 \times 10^5$	$\beta = 2.55$ $a/b = 15$	$s_0 = 5.7 \times 10^{-13}$ $[\eta] = 0.65$; $\eta_0 = 1.1 \times 10^{-2}$
S	4.5	$M = 3 \times 10^5$	$\beta = 1.94 \times 10^6$ $a/b < 1$	$\rho = 1.126$; $\bar{\nu} = 0.59$ $M = 2 \times 10^5$ (PPS_N fraction 2)
F	0.06	Flory constant $= 2.5 \times 10^6$	$M = 1.3 \times 10^6$	
F	4.5	Flory constant $= 2.5 \times 10^6$	$M = 2.06 \times 10^5$	
K	0.06	$L/b = 20$ $b = 25$ mμ	$M_0 = 2.1 \times 10^5$	
K	4.5	$L/b = 15$ $b = 12.5$ mμ	$M_0 = 2.1 \times 10^5$	

[a] Molecular weight and shape parameters using various theoretical approaches and experimental data. Equation S (Scheraga and Mandelkern, 1953): $\beta = N[\eta]^{1/3}\eta_0 s_0/M^{2/3}(1 - \bar{\nu}\rho)$; a/b = axial ratio; eq F (Flory, 1953): $M^{2/3} = N[\eta]^{1/3}\eta_0 s_0/(2.5 \times 10^6)(1 - \bar{\nu}\rho)$; eq K (Kirkwood and Auer, 1951): $M_0 = (\pi N/2250[\eta])[L^2 b/\ln(L/b)]$.

The data at high Γ give values that are higher than, but near those usually chosen for protein–polysaccharides. For this reason, it is felt that use of $[s_x]$ and $[\eta_x]$ obtained at high ionic strength will allow calculation of more realistic molecular weights if a value for β between 2.5 and 3 is used. Both the Flory and Kirkwood models give reasonable agreement with the experimental data providing appropriate assumptions are employed. See Table II. The best method, however, remains the sedimentation equilibrium experiment since the correction and ambiguities noted above are not necessary. The value of β calculated at high Γ leads to an estimated axial ratio of about 30. This indicates that the molecules aggregate into rodlike structures, but that the detailed structure of the monomers is still unknown.

The evaluation of the data by the two-species and three-species plot techniques is consistent with the presence of a monomer species and some higher polymers of that species or with discrete heterogeneity.

Since the C-4S chains themselves are essentially homogeneous with $\bar{M}_z/\bar{M}_w$ of 1.04 (Marler and Davidson, 1965) and since the peptide core represents only about 10% by weight of the proteoglycan, the observed dispersity in molecular size may arise from peptide fragments of differing size containing different numbers of covalently linked saccharide chains. Thus, a model of this system would have species of molecular weight approximately 14,000 (peptide-C-4S), 29,000 [peptide-(C-4S)$_2$], 45,000 [peptide-(C-4S)$_3$], etc. Superimposed on this is the possibility of association which may or may not be readily reversible. The nature of the aggregates formed as a result of such association would be dependent on concentration, ionic strength, and possibly the composition (*e.g.*, charge density) of the species involved.

A number of fractionation techniques have been employed in an effort to better define the nature of the proteoglycan system. The results do not clearly permit us to distinguish between the possibilities indicated above since, for example, fractions isolated after gel filtration chromatography exhibit substantially similar sedimentation equilibrium properties apparently independent of elution volume. It seems reasonable to suggest that a protein core of discrete size is the initial biosynthetic product to which are added saccharide units until an appropriate chain size is reached. Secretion to the extracellular space may terminate further polymer assembly and the material isolated may be a composite of new and old proteoglycan reflecting some proteolysis. The possibility of aggregation is consistent with the data reported and does not require a protein core of abnormal length to account for some observed molecular weights. The general range of molecular weights reported in this paper using sedimentation equilibrium are considered by us to be thermodynamically valid. Some previously reported molecular weights calculated using sedimentation velocity and intrinsic viscosity studies appear too high since only studies done at high ionic strength and employing high β values (2.5–3.0) yield values consistent with those obtained by equilibrium sedimentation.

References

Ansevin, A. T., Roark, D. E., and Yphantis, D. A. (1970), *Anal. Biochem. 34*, 237.

Bitter, T., and Muir, H. M. (1962), *Anal. Biochem. 4*, 330.

Blix, G., and Snellman, O. (1945), *Ark. Kemi. Min. Geol. 19A*, No. 32.

Casassa, E. F., and Eisenberg, H. (1960), *J. Phys. Chem. 64*, 753.

Casassa, E. F., and Eisenberg, H. (1961), *J. Phys. Chem. 65*, 427.

Dunstone, J. R., and Franek, M. D. (1969), *J. Biol. Chem. 244*, 3654.

Flory, P. J. (1953), Principles of Polymer Chemistry, Ithaca, N. Y., Cornell University Press.
Gerber, B. R., Franklin, E. C., and Schubert, M. (1960), *J. Biol. Chem. 235*, 2870.
Kirkwood, J. G., and Auer, P. L. (1951), *J. Chem. Phys. 19*, 281.
Lloyd, A. G. (1959), *Biochem. J. 72*, 133.
Luscombe, E. M., and Phelps, C. W. (1967), *Biochem. J. 102*, 110.
Malawista, I., and Schubert, M. (1958), *J. Biol. Chem. 230*, 535.
Marler, E., and Davidson, E. A. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 648.
Mathews, M. B. (1956), *Arch. Biochem. Biophys. 61*, 367.
Mathews, M. B., and Dorfman, A. (1953), *Arch. Biochem. Biophys. 42*, 41.
Mathews, M. B., and Lozaityte, I. (1958), *Arch. Biochem. Biophys. 74*, 158.
Mejbaum, W. (1939), *Z. Physiol. Chem. 258*, 117.
Muir, J., and Jacobs, S. (1967), *Biochem. J. 103*, 367.
Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 164*, 245.
Sajdera, S. W., and Hascall, V. C. (1969), *J. Biol. Chem. 244*, 77.
Scatchard, G. (1946), *J. Amer. Chem. Soc. 68*, 2315.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179.
Simpson, D. L., Hranisavljevic, J., and Davidson, E. A. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1278.
Tanford, C. (1955), *J. Phys. Chem. 59*, 798.
Tanford, C., Marler, E., Jury, E., and Davidson, E. A. (1964), *J. Biol. Chem. 239*, 4034.
Yang, J. T. (1961), *Advan. Protein Chem. 16*, 323.

Evidence for the Presence of Two Nonidentical Subunits in Carbamyl Phosphate Synthetase of *Escherichia coli*†

Susan L. Matthews and Paul M. Anderson*

ABSTRACT: A preparation of carbamyl phosphate synthetase which appears to be homogeneous by acrylamide gel electrophoresis can be obtained by carrying out as the last purification step gel filtration on Sephadex G-200 twice, once in presence of UMP (enzyme existing as a monomer) and then again in the presence of ornithine (enzyme existing as oligomer). Two protein peaks are obtained when carbamyl phosphate synthetase is subjected to gel filtration on Sepharose 4B in the presence of 6 M guanidine hydrochloride. Estimation of their molecular weights by relating their elution volumes (distribution coefficients) with those of proteins of known molecular weight gave values of 145,000 and 48,000 for the large and small peaks, respectively (referred to as the α and β subunits, respectively). Summation of these two molecular weight values yields a value which is consistent with the range of 170,000 to 200,000 established previously for the molecular weight of this enzyme. The ratio of protein in the two peaks is 2.7 to 1; the amino acid compositions of the protein in the two peaks are significantly different, but summation of the amino acid compositions, assuming that the enzyme is composed of one of each of the two subunits with molecular weights of 140,000 and 48,000, respectively, yields values very close to those of the native enzyme. The presence of two nonidentical subunits was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; two protein bands are obtained, the slower and faster moving bands corresponding to the α and β subunits, respectively. Estimation of the molecular weight of the two components corresponding to the α and β subunits by relating their relative mobilities with those of proteins of known molecular weight gave values of 138,000 and 48,000, respectively. Previous studies have shown that at least three different reactive SH groups are present in carbamyl phosphate synthetase and that the availability of two of these SH groups for reaction with *N*-ethylmaleimide is dependent on the presence or absence of different ligands. Reaction of these SH groups individually with *N*-[^{14}C]ethylmaleimide gave the following results. (1) The single SH group which reacts under all conditions and which is the only SH group which reacts when ornithine is present is located in the α subunit. (2) The SH group which reacts only when ATP–$MgCl_2$ and bicarbonate are present (resulting in a 50% loss in the synthetase and ATP synthesis activities) is located in the B subunit. (3) The SH group which can be reacted with *N*-[^{14}C]ethylmaleimide after the above two SH groups have been reacted with *N*-ethylmaleimide (resulting in nearly complete loss of synthetase activity, but a 100% increase in ATP synthesis activity) is located in the α subunit.

At least three different SH groups can be identified in carbamyl phosphate synthetase from *Escherichia coli* B by showing that the availability of each for reaction with *N*-ethylmaleimide (NEM)[1] or DTNB is dependent on the presence (or absence) of different substrates or allosteric effectors and that different effects on the catalytic activities of the enzyme are observed as a result of reaction of each of the differ-

† From the Department of Chemistry, Southern Illinois University, Carbondale, Illinois 62901. *Received October 19, 1971.* This work was supported in part by U. S. Public Health Service Grant AM11443. A preliminary account of this work has appeared (Anderson *et al.*, 1970a).

* To whom correspondence should be addressed. Present Address: Medical Education Program, University of Minnesota-Duluth, Duluth, Minnesota 55812.

[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; Gdn·HCl, guanidine hydrochloride.