showing α_η^3/α_s^3 against N at fixed α_s^3. Also the results of case (c) for $\beta = \beta_0\alpha$ and the KY limit are reported. Since most of the experimental data in the literature fall in the range between the KY limit and 0.8, besides case (c) with $\beta = \beta_0\alpha$, only case (b) with $\beta = \beta_0$ permits the interpretation of experiments (case (c) with $\beta = \beta_0$ would in fact be lower than curve b′ in the figure and thus be outside the field of interest).

Although it seems reasonable to assume β to be dependent on the expansion, we think that viscosity and dimension data accurately corrected for polydispersity and temperature (or solvent) dependence of the unperturbed dimensions should permit distinguishing which one of the two approximations for β is the nearest to the real condition.

In conclusion, we have shown that the Fixman results for perturbed $[\eta]$ do not agree with the experimental data, nor with Kurata–Yamakawa predictions, owing to the poor approximation employed to calculate the hydrodynamic interaction. When an evaluation of the hydrodynamic interaction made on the basis of the Kurata–Yamakawa assumption of the excluded volume is introduced into the Fixman theory, results that agree with those of KY are obtained in the nondraining limit. The critical role of the appropriate formulation of the hydrodynamic interaction is tested by the differences found between uniform and nonuniform approximations. A further improvement is obtained by correctly reformulating the theory for partial draining; in this case the experimental results can be explained in terms of changes in molecular weight or draining of the polymer chains.

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Charge-Induced Conformational Changes in Carboxymethylamylose[1]

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ABSTRACT: Viscometric and potentiometric proton titrations of carboxymethylamylose (CMA) have been conducted in aqueous salt solution in the presence and absence of the cosolvent *n*-butyl alcohol (BuOH). The dependence of the intrinsic viscosity $[\eta]$ and the logarithmic apparent acid dissociation constant pK_a on the degree of ionization α have been analyzed for evidence of skeletal conformation changes induced by changes in the linear charge density ρ of the weak polyelectrolyte. The results suggest a model for aqueous CMA in the presence of saturating amounts of BuOH which envisages stabilization by BuOH of helical sequences in the polymer chain at sufficiently low ρ; in the same medium at higher ρ the polymer is prevented from forming helical sequences by strong intramolecular electrostatic interactions. Even at low ρ the development of helical character in this medium is not sufficient to produce the dramatic phenomena in plots of $[\eta]$ and pK_a vs. α characteristic of polypeptides known to undergo charge-induced transformations from helix to coil. Data gathered in the absence of BuOH suggest that CMA at low ρ in aqueous salt solution contains numerous compact residue sequences as predicted by earlier theoretical considerations of the configuration of aqueous amylose.

The configuration of amylose in aqueous solution has been the subject of extensive investigation. Amylose was the first natural polymer for which a helical structure was recognized in the solid state;[3] whether a helical conformation is retained in aqueous solution is a matter of continuing debate.[4–9] Amylose has proven to be less amenable to studies of its aqueous solution configuration than certain other biopolymers, e.g., polypeptides and polynucleotides, because (1) no spectral property of the uncomplexed macromolecule which is unambiguously sensitive to polymer conformation has been discovered,[10–12] and (2) unmodified amylose forms a metastable aqueous solution which is subject to crystalline phase separation (retrogradation) at a rate that depends upon the chain-length distribution of the sample[8] and the pH.[13] The first limitation has restricted definitive studies of the aqueous solution configuration to light scattering and hydrodynamic measurements. The second difficulty has led many workers to study amylose in more potent solvents such as dimethyl sulfoxide,[14] formamide,[15] and ethylenediamine[16] or in aqueous medium at pH values greater than about 12 under which conditions amylose becomes a polyelectrolyte via dissociation of hydroxyl protons.[17] Other groups have countered the aqueous solubility difficulties by investigating the properties of amylose derivatives, e.g., carboxymethylamylose and diethylaminoethylamylose, more conveniently soluble in aqueous media.[4,18,19] An additional advantage attends studies of weak polyacid derivatives in that the chain configuration can be manipulated by bringing about changes in degree of ionization.

Notwithstanding the limitations to obtaining stable neutral aqueous solutions of unmodified amylose, several investigators have gathered apparently reliable experimental results from studies in this medium following special procedures to dissolve the polymer. Studies in which the dependence of intrinsic viscosity $[\eta]$ on degree of polymerization (DP) has been determined[20-23] suggest that neutral aqueous media are ideal solvents for unsubstituted amylose, i.e., the exponent in the Mark–Houwink equation is reported to be very close to 0.5 over a wide range of DP. Although conclusive confirmatory evidence that such systems are at the Flory Θ condition[24] is lacking,[25] it is generally accepted that amylose in neutral aqueous media exhibits properties unperturbed by long-range volume exclusion. Whereas the observed modest dependence of $[\eta]$ upon DP clearly precludes a rigidly helical amylose molecule in aqueous solution, chains possessing short-range or disrupted helical structure or wormlike, flexible helical chains cannot be ruled out on the basis of these hydrodynamic data, and indeed such models may be consistent with the observed Gaussian random coil behavior.[25] Thus, within the limitations imposed by these results, a variety of models have been proposed for the amylose chain in aqueous solution including a segmented or interrupted helix,[9,26] a wormlike coil with defined helical backbone,[8,27] and a random coil possessing no residual helical character.[6,7]

All of the chain models proposed have been based on additional results which supplement the basic hydrodynamic data just reviewed. An important source of further evidence relevant to the configuration of amylose in water is in the effect on the macromolecule of the presence of what may be termed helix-promoting or helicogenic agents. Addition of such compounds, most conspicuously iodine in the presence of iodide (I_2/I^-) and normal butanol (BuOH), can cause the precipitation of amylose from aqueous solution in the crystalline helical modification known as V-amylose. The crystalline V-amylose precipitated by several helix-promoting molecules is known to contain the helicogenic agent complexed within the annular cavity of the helix.[28] The driving force for this interaction is unknown at present and may very well differ among the several helicogenic substances. It may be presumed that such helicogenic cosolvents enhance the stability of the V-amylose conformation in aqueous solution, irrespective of whether or not V-helix constitutes a stable conformation in the absence of cosolvent. It should be noted that retrograded amylose, which precipitates spontaneously from aqueous media in the absence of helicogenic substances, adopts the B-amylose conformation rather than the V.[5] Both the crystalline helices of V- and B-amylose are thought to be characterized by about six glucose residues per helical turn, but the V modification is the more compact and displays an axial translational per residue of 1.33 Å, whereas that for B-amylose is 1.73 Å.[5]

Viscometric titrations of aqueous amylose[7,9,26] and of carboxymethylamylose[18] with both I_2/I^- and BuOH have been reported. The most recent and complete study is that of Senior and Hamori.[9] These authors have presented a thorough review of the earlier literature. We repeat here only the reported observation that addition of both I_2/I^- and BuOH to aqueous amylose produces a decrease in the intrinsic and/or reduced specific viscosity of the polymer. These results, coupled with certain kinetic data on the aqueous amylose–I_2/I^- system,[29] have led Senior and Hamori to espouse a broken helical model for aqueous amylose in which the helical sequences are more extended, and presumably less rigid, than the V-amylose helix of the amylose–I_2/I^- complex. The helical sequences are postulated by Senior and Hamori to be joined by "shorter random-coil sections." Banks and Greenwood[7] have interpreted the results of the viscometric titrations as evidence for an amylose configuration in neutral aqueous media which they describe as "a statistical, or random, coil having no helix content". An earlier conclusion from viscometric titrations and spectroscopic study of amylose–I_2/I^- binding isotherms due to Szejtli and coworkers[26] holds that the aqueous amylose chain is essentially a jointed or periodically disrupted V helix. The evidence leading to this conclusion has been refuted by Banks and Greenwood[7] and by Senior and Hamori.[9]

Spectroscopic studies of amylose–I_2/I^- binding represent another kind of investigation which has led workers to propose models for the chain configuration of aqueous amylose. It is well to remark, however, that spectroscopic data such as those for the I_2/I^- complex of amylose, which characterize only the *product* of the ligand binding process, cannot distinguish binding mechanisms which involve induction of helical binding sites by the ligand from those in which the ligand binds to sites within a preexisting helical cavity. Without supplementary data such studies cannot disclose the chain configuration in the absence of ligand. Recently, Pfannemueller and her coworkers[8] have concluded from studies of the chain-length dependence of optical rotatory dispersion and circular dichroism of the amylose–I_2/I^- complex together with studies of the dependence on chain length of the rate of amylose retrogradation from aqueous solution that high molecular weight amylose is "a wormlike helical chain" in water. Rao and Foster[27] proposed a similar chain model on the basis of hydrodynamic studies in neutral aqueous solution and in the pH range 10–14.

The behavior of amylose in alkaline solution has been interpreted in several ways, although experimental results have been relatively consistent. Some of the earlier data[27] were rendered obscure by failure to maintain constant ionic strength with addition of base, but measurements of the pH dependence of $[\eta]$ in the presence of excess salt reveal a minimum in $[\eta]$ at pH 12.[17,30-32] Initially this minimum was thought to indicate a disruption, provoked by the onset of ionization of hydroxyl groups near pH 12, of an extended helical structure, followed by polyelectrolyte expansion of the chain at still higher pH.[27] More recently it has been suggested, by analogy to the effect on $[\eta]$ of helix-promoting agents, that the reduction in viscosity near pH 12 corresponds to the formation from randomly coiled amylose of helical structure stabilized by "ionic bonding" involving pairs of ionized hydroxyl groups linked by intervening sodium ions.[17] It is interesting to observe in this context that titration of alkaline aqueous amylose solutions (0.01 *M* KOH with no supporting electrolyte) with BuOH produces a larger diminution of the reduced specific viscosity of amylose than occurs in neutral aqueous solution.[33]

An alternative line of reasoning which has been employed to provide a description of the solution configuration of amylose involves attempts to fit quantitative experimental data on the configuration-dependent solution properties[34] of amylose using a detailed geometric model of the polymer skeleton in conjunction with empirical conformation energy calculations and the statistical mechanical theory of polymer chain configuration.[35,36] These methods were first applied to amylose by Rao and collaborators[37] and subsequently by Brant and coworkers.[5,6] A statistical mechanical treatment[6] applicable to amylosic chains in neutral aqueous media was able to provide a quantitative fit to the observed chain length and temperature dependences of the unperturbed chain dimensions of carboxymethylamylose and diethylaminoethylamylose in aqueous salt solutions at high ionic strength.[4] Detailed interpreta-

tion of the chain model invoked led Brant and Dimpfl[6] to describe the chain as a statistical (random) coil without residual helical character in agreement with the earlier conclusions of Banks and Greenwood.[7]

Chain models based on this approach, because they are grounded on fundamental molecular properties such as chemical bond lengths and bond angles and potential functions for changes in local skeletal conformation, can render more specific such generic descriptions of the chain configuration as "random coil" or "disrupted helix". To facilitate considerations below of the present experimental results in terms of the several chain models which have been proposed for aqueous amylose we attempt briefly in what follows to illuminate our understanding of these generic terms. Thus, if the bond angles and bond lengths characterizing the amylose skeleton are assumed constant and the glucopyranose ring is taken to be fixed in a single conformation, then the mutual orientation of an adjoining pair of glucose residues i and $i + 1$ is governed solely by the torsion angles φ_i and ψ_i about the $C(1)_i$–$O(1)_i$ and $O(1)_i$–$C(4)_{i+1}$ bonds of the glycosidic linkage.[5,38] A regular helical chain conformation may be generated by requiring that a set of values adopted by a given pair of angles φ_i,ψ_i be repeated in all such angle pairs in the chain.[5,39] A given residue $i + 1$ in the chain can be said to adopt a *helical state* if, over a period long compared with the period of bond torsion in a disaccharide, the torsion angles φ_i,ψ_i retain values characteristic of some particular helical conformation.[39] A consecutive array of glucose residues having identical values φ_i,ψ_i may be defined as a *regular helical sequence;* glucose residues whose conformations are not so restricted are said to be in the *random coil state,* and these in consecutive array comprise a *random coil sequence.* A random coil chain then comprises a single random coil sequence, and a regular helical chain likewise consists of just one regular helical sequence. In these terms we interpret a *wormlike sequence* to be one in which small amplitude deviations may occur in each residue about a set of mean values φ_i,ψ_i which characterize a particular helical state. A wormlike chain is then understood to comprise a single wormlike sequence. Evidently the wormlike chain model occupies a continuum between the regular helical chain and the random coil. We believe, however, that the model is usually intended to refer to chains subject only to small fluctuations from a set of mean values φ_i,ψ_i such that the helical character of the skeletal trajectory is preserved. This is our interpretation of the chain models proposed by Pfannemueller et al.[8] and by Rao and Foster.[27]

Whenever experimental evidence warrants the differentiation between helical and coil states for the residues of a given chain with the consequent occurrence in the chain of both helical (including wormlike) and coil sequences, it may then be appropriate for statistical mechanical treatments of the chain to partition the conformation space of a given residue into two regions designated "helix" and "coil".[39,40] This approach has produced successful theoretical treatments of the configurational properties of the polypeptides[40,41] and is a potentially valuable approach to description of the amylose chain, particularly in the presence of helicogenic cosolvents.[25,42] We interpret the aqueous amylose chain models proposed by Szejtli and coworkers[26] and by Senior and Hamori[9] to belong to this category in which the residues are seen to occur in one or the other of two distinguishable classes. Szejtli et al. partition the residues between random coil and regular V-helical states whereas Senior and Hamori envisage the occurrence along with random coil sequences of flexible wormlike sequences which are on the average more extended than segments of V-helix of the same DP.

It is our purpose in the work reported here to consider the effect on chain configuration of increasing degree of dissociation α of the carboxymethylamylose (CMA) chain. In view of the interesting charge-induced conformation changes observed in some other polyelectrolytes, e.g., polymethacrylic acid (PMA),[43] butyl vinyl ether–maleic acid copolymer (BVE-MA),[44] poly(2-vinylpyridine),[45] and, particularly, poly(L-glutamic acid) (PLGA)[46,47] and poly(L-lysine) (PLL),[48] the effect of polyion charge on the properties of CMA is of special interest. The principal emphasis in the current studies is on viscometric and potentiometric proton titrations of CMA with acid and base in aqueous salt solution in the presence and absence of BuOH. Investigations were conducted on CMA samples possessing degrees of substitution (DS) in the range 0.09–0.55. Spectrophotometric measurements of iodine binding capacity were carried out at low α in order to probe the nonelectrostatic effects of carboxymethyl substitution on the ease of helix formation. The dependence of iodine binding on α was also investigated as was the potentiometric titration behavior of CMA in the presence of I_2/I^-. Results obtained in the present studies are considered in light of viscometric and potentiometric titration data on other well-characterized weak polyelectrolytes, and an interpretation of the results is offered in terms of the several chain models for aqueous amylose which have been reviewed above.

Experimental Section

Preparation of Carboxymethylamylose. The majority of the measurements reported here were made with an unfractionated sample of CMA prepared by Min[25] and hereinafter known as CMA-V. The sample was characterized by DS = 0.55 ± 0.03 and a number average DP = 600 ± 30. Several samples of lower DS, CMA-I, -III, -III, and -IV, were prepared as described below.

A commercial sample of potato amylose, Superlose (Stein, Hall, and Co., Inc., N.Y.), was purified by repeated recrystallization of the BuOH complex from water following a procedure described earlier.[4] Four successive recrystallizations of the original material yielded a sample with an iodine binding capacity of 20 ± 1 g of I_2/100 g of amylose as measured by amperometric titration.[4] This is the iodine binding capacity usually associated with amylose samples free of branched chain starch components[49] and is to be compared with 17.5 g of I_2/100 g of amylose observed for the starting material.[4]

The wet amylose–BuOH complex, typically weighing about 300 g and containing some 5% amylose by weight, was dissolved in 2 l. of 0.5 *M* NaOH, through which nitrogen had been bubbled, by stirring a 3-l. flask. Concentrated NaOH was added to make the solution 4 *M* in base; the solution was kept under nitrogen to prevent oxidative degradation. The flask was transferred to a water bath at 55°C and from 40 to 210 g of chloroacetic acid was added. The amount of chloroacetic acid added was in proportion to the desired DS as was the reaction time which varied from 3 to 83 hr. The reaction was terminated by transfer of the flask to an ice bath and neutralization by gradual addition of 12 *N* HCl. After filtration through a coarse sintered glass funnel the polymer solution was dialyzed exhaustively against distilled water until, after several days, the presence of Cl^- could not be detected in the external solution. It was then filtered through a medium sintered glass funnel and freeze dried.

Characteristics of the several amylose derivatives and the respective preparative procedures are presented in Table I. The DS value was determined by titration with NaOH and dry weight analysis of the acid form of the polymer, obtained by cation exchange as described below.[25] The iodine binding capacity of amylose may be measured readily by amperometric titration with coulometric iodine generation.[4] Because of the lower binding affinity of the derivatives, the binding capacity of CMA was determined from spectrophotometric titration with iodine as described below. Viscosity average DP's were estimated from intrinsic viscosities measured in solvents for which appropriate Mark–Houwink equations are reported.[4,7]

Potentiometric Proton Titration. In addition to providing the relationship between pH and α required for the determination of the depennce on α of various measured quantities, titration data

Table I
Preparation and Characterization of CMA Samples

Sample	Mass of $ClCH_2COOH$, g	Reaction time, hr	DS	$\overline{DP}[\eta]$	I_2 binding capacity, g of I_2/100 g of amylose	$\langle r^2\rangle/xl^2$
A[a]			0		21	5.1[b]
I	40	3.2	0.092	1610[c]	21	
II	55	16	0.12	1500[c]	21	5.3
III	150	17	0.31	1850[c] 1800[d]	20	
IV	210	83	0.54			
V			0.55	2100[c]	18 ± 3	7.4[e]

[a] Amylose. [b] Characteristic ratio in neutral aqueous medium.[7] [c] Determined from Mark–Houwink relation for amylose in 0.15 *M* KOH.[7] [d] Determined from Mark–Houwink relation for CMA of DS = 0.3 in 0.78 *M* NaCl at pH 7.[4] [e] In agreement with this result Goebel and Brant[4] estimate $C_\infty = 8.0 \pm 0.5$ for CMA-V in 0.5 *M* NaCl from the DP, $[\eta]$, and osmotic second virial coefficient measured in that medium.

were employed to measure polymer concentrations by a procedure described below. Intrinsic interest in titration data attaches to analysis of hydrogen ion titration curves in the form pK_a (apparent acid dissociation constant) vs. α, which are particularly revealing for polyacids undergoing marked charge-induced conformational transitions.[43-48]

Titrations were performed on CMA samples in the fully protonated form (HCMA) that were prepared as follows. Solutions of the freeze-dried polymer, present predominantly as the sodium salt (NaCMA), were stirred with excess Amberlite IR-120 CP cation exchange resin which had been equilibrated with 1 *N* HCl and then washed repeatedly with distilled water until the pH of the rinse was above 5.5. Fresh portions of resin were added to the polymer solution until a constant pH value was attained, indicating complete conversion of NaCMA to HCMA. Filtration through a 0.8 μ Millipore filter removed the resin and other particulate material. The required amounts of water and other components, e.g., BuOH and NaCl, were then added to bring the solution to the desired concentration of polymer and other solute species. The pH titrations were performed on aliquots of such solutions under nitrogen at 22 ± 1° using a Beckman Expandomatic pH meter and Sargent S-30072-15 combination electrode. The precision of the pH measurements was ±0.01 pH units, and the meter drift was within 0.005 pH units hr^{-1}. The meter was standardized in all cases using commercial buffers dissolved in boiled, distilled water. Titrants (Harleco), 0.2000 *N* HCl and NaOH, were delivered with Gilmont 0.2-ml syringe microburets. A solvent blank identical with the sample except for the presence of polymer was titrated immediately afterwards.

The degree of dissociation α at a given measured pH was determined from the titration data using equations derived previously.[4] Equations 1, 2, 6, 6′, 7, and 7′ of ref 4 are applicable in the present case, providing the primed concentrations which appear in those equations are understood to refer to the solvent in the present case, i.e., aqueous 0.05 *M* NaCl in the presence and absence of BuOH, and the unprimed concentrations to the polymer solutions prepared using these solvent systems. Note that eq 6′ of ref 4 is formulated in terms of the degree of *ionization* β which for polycarboxylic acids may be identified with α. Since the titrations were carried out on solutions of HCMA prepared as described above, the concentrations C_{Na} and C_{Cl} of sodium and chloride ions in the polymer solutions before titration were identical. Under these conditions eq 2 of ref 4 adopts the form $\beta_0 = (C_H - C_{OH})/C_0$, so that the first two terms on the right-hand side of eq 6′ vanish to yield eq 1, which relates α to the molar concentrations $[H^+]$ and $[OH^-]$ in a polymer solution, originally of volume V_0 and with equivalent molar concentration of polymer C_0, which has been titrated with a volume v of *C M* NaOH solution to a given pH. Equation 1 is not

$$\alpha = \frac{Cv}{C_0V_0} + \frac{V_0 + v}{C_0V_0}([H^+] - [OH^-]) \quad (1)$$

satisfactory for calculation of α because the relationship of measured pH to $[H^+]$ and the apparent ion product of water required to obtain $[OH^-]$ are not known. However, if it is assumed, as it may be for solutions sufficiently dilute in polymer, that these required relationships are the same for the polymer solution as for the solvent at the same pH, then titration data for the solvent may be used to eliminate the quantity $([H^+] - [OH^-])$ in eq 1. That is, a volume V_0 of solvent may be titrated with V' l. of HCl or v' l. of NaOH, both at a concentration *C M*, to a given pH for which it is desired to evaluate α for the polymer solution. Equations 7 and 7′ of ref 4 provide, respectively, expressions for $([H^+]' - [OH^-]')$ for the solvent titrated in this manner with HCl or NaOH. Substitution from eq 7 and 7′ of ref 4 into eq 1 yields respectively

$$\alpha = \frac{Cv}{C_0V_0} + \frac{V_0 + v}{C_0V_0}\left[\frac{V_0(C_{H'} - C_{OH'})}{V_0 + V'} + \frac{CV'}{V_0 + V'}\right] \quad (2)$$

$$\alpha = \frac{Cv}{C_0V_0} + \frac{V_0 + v}{C_0V_0}\left[\frac{V_0(C_{H'} - C_{OH'})}{V_0 + v'} - \frac{Cv'}{V_0 + v'}\right] \quad (2')$$

Since the solvent is nearly neutral, the respective initial concentrations $C_{H'}$ and $C_{OH'}$ of hydrogen and hydroxide ions are small, and the first term in brackets is negligible. Recognizing that $C_0V_0 = Cv_{eq}$, where v_{eq} is the volume of *C M* NaOH required to titrate the polymer solution to the equivalence point, and choosing experimental conditions which render $V_0 \gg v, v', V'$, eq 2 and 2′ reduce respectively to the practical working relationships

$$\alpha = (v + V')/v_{eq} \quad (3)$$

$$\alpha = (v - v')/v_{eq} \quad (3')$$

These equations are equivalent to those employed without detailed explanation by other workers.[44a,46]

Using this procedure to establish the dependence of α on pH, an experimental uncertainty of ±0.01 in pH corresponded to an uncertainty of ±0.01 in α. The method employed here to standardize the pH meter renders particularly uncertain the absolute meaning of the measured pH in solutions containing BuOH,[50] but the above procedure for establishing the value of α at any given measured pH is independent of the method of standardization, provided the meter is standardized consistently for titrations of polymer solution and solvent.

Preparation of Polymer Solutions for Intrinsic Viscosity Determinations. Intrinsic viscosities were measured as a function of α for samples of CMA-II and -V in 0.05 *M* NaCl in the presence or absence of 6% BuOH. (This concentration of BuOH, defined as 60 g of BuOH per liter of solution, nearly saturates the 0.05 *M* aqueous NaCl and is typical of BuOH concentrations used to precipitate crystalline V-amylose.) Freeze-dried NaCMA-V was dissolved in pure water and filtered successively through 0.8 and 0.45 μ Millipore filters; the NaCMA-II was dissolved in ca. 0.05 *M* NaOH, filtered as above, and then neutralized with HCl. Appropriate amounts of NaCl and water were added to make the final solutions 0.05 *M* in NaCl. Polymer concentrations ranged from 0.05 to 0.25 g dl^{-1}. These stock solutions were refrigerated and used for periods of up to 2 months. No indication of polymer degradation, as evidenced by decrease in viscosity, could be found.

Solutions for intrinsic viscosity measurements in the absence of BuOH were prepared by dilution of the stock solutions with 0.05 *M* NaCl and subsequent adjustment of the pH with 0.2 *N* HCl or NaOH to achieve the desired α as determined from the titration curve of HCMA-II or -V in 0.05 *M* NaCl. Approximately 15 ml of this polymer solution, possessing a relative viscosity in the range 1.1–1.3 at a chosen pH, was dialyzed overnight against 80 ml of 0.05 *M* NaCl adjusted to the same pH. Solutions for intrinsic viscosity measurements with 6% BuOH present were prepared by the same procedure except that 7.12% BuOH was added to the external solution (dialyzate) prior to dialysis to produce an over-all concentration of 6% BuOH in the system at dialysis equilibrium. When BuOH was present, the required relationship between pH and α was obtained from the titration curve of the appropriate

HCMA in 0.05 M NaCl containing 6% BuOH. In establishing the pH vs. α titration curves the unequal distribution of BuOH and/or NaCl across the dialysis membrane was neglected, and titrations were conducted under the mean concentration conditions, i.e., 6% BuOH and 0.05 M NaCl, as though these concentrations obtained in the dialyzed polymer solutions. Given the high molar ratio of BuOH to glucose residues (>150), the assumption of equal distribution of BuOH is justified despite the presumed preferential binding of BuOH by CMA. Likewise, neglect of the Donnan effect on NaCl distribution is justified in view of the mobile ion concentration in relation to the equivalent concentration of polymer (≤0.004 equiv $l.^{-1}$).

Intrinsic Viscosity Measurements. Flow times of an aliquot of the dialyzed polymer solution, of successive dilutions of the dialyzed polymer solution made by volumetric addition to dialyzate, and of dialyzate alone, were measured in a Cannon-Ubbelohde size 50 dilution viscometer at 25 ± 0.02°C. A mean deviation of less than 0.1 sec from the mean of at least three measurements was required. Solvent flow times in the esence and absence of BuOH were ca. 195 and 257 sec, respectively, and no kinetic energy corrections were made. Measurements with CMA-V at high pH, i.e., the most highly extended polymer of this study, revealed a very small shear rate dependence which was subsequently neglected. The value of α was determined from the average of the pH value of the dialyzed polymer solution immediately following dialysis and that of the last dilution in the viscometer. The uncertainty in α arising from a difference between those pH values never exceeded ±0.02 α units.

Concentration Determination for Intrinsic Viscosity Measurements. An aliquot of the dialyzed polymer solution of volume V_0 was titrated with 0.2 N NaOH and/or 0.2 N HCl, and the volumes v of base and/or V of acid required to reach pH values corresponding to specified values of α, e.g., 0.25, 0.50, 0.75, were recorded, these pH values being known from α vs. pH titration data established as described above for polymer in the same medium. A corresponding sample of dialyzate, also of volume V_0, was likewise titrated to the same set of pH values, and the required volumes v' of base and/or V' of acid were recorded. These data are related to the equivalent concentration C_0 of polymer, in equivalents of polymeric proton binding groups per unit volume of solution, by eq 8 and 8′ of ref 4. Considering, for example, the case of titration of dialyzed polymer solution and dialyzate with aid between pH values corresponding to α_1 and α_2, eq 8 yields

$$C_0 = \frac{-C}{V_0(\alpha_2 - \alpha_1)}[(V - V')_2 - (V - V')_1] \quad (4)$$

where β of eq 8 has been replaced by α and C is the equivalent concentration of the acid titrant and where $(V - V')_i$ is the difference in titrant volumes required to bring the polymer solution and dialyzate to the pH value corresponding to α_i. Equations 8 and 8′ can also be combined to yield an expression for C_0 analogous to eq 9 of ref 4. Coupled with a knowledge of the equivalent weight of the polymer, this determination of C_0 enables one to compute the weight concentration of polymer, which is reported here as C_{ps} in g dl^{-1} of the sodium salt, NaCMA. The equivalent weight M_{ps} of the pure sodium salt can be computed from the known DS using $M_{ps} = M_u/DS$ and $M_u = M_r + DS(M_s - 1)$, where M_u is the mean weight per polymer repeat unit, $M_r = 162$ is the weight of the unsubstituted glucose residue, and $M_s = 81$ is the weight of the $-CH_2-COONa$ substituent group.[4]

In addition to the dialyzed polymer solution an aliquot of the last dilution in the viscometer was titrated in similar fashion to determine its polymer concentration. Because of the small concentrations involved in this case, agreement in concentrations determined using titration data over several different 0.25 increments in α was generally only within ±2–5%. However, the averaged values of C_{ps} obtained by titration over several ranges of α for the dialyzed polymer solution and for the final dilution, respectively, agreed to within ±2%, resulting in a similar degree of confidence in the intrinsic viscosity values, since uncertainty in polymer concentration is the principal source of error in the measured value of $[\eta]$.

Spectrophotometric Titrations. Spectrophotometric measurements were made with a Cary 14R recording spectrophotometer using matched 1-cm pathlength quartz cells at 22 ± 1°C. Polymer samples were titrated (1) with I_2 in the presence of I^- at low α to determine the iodine binding capacities of the several CMA samples, and (2) with HCl and NaOH in the presence of I_2 and I^- to determine the dependence of the stability of the CMA–I_2/I^- complex on α.

In titrations of type (1) optical density (OD) measurements were made at 600 nm on CMA dissolved in aqueous 0.05 M KI as a function of added I_2. A 3.00-ml polymer solution was titrated directly in the spectrophotometer. Titrant, 1.80×10^{-3} M I_2 dissolved in aqueous 0.05 M KI, was delivered with a Gilmont 0.2 ml syringe microburet through a Teflon delivery tube which entered the spectrophotometer cell through a Teflon cap bored to accommodate the tube. Stirring was accomplished with a Teflon coated magnetic stirring bar in the cell and a magnetic stirring motor located in the cell compartment beneath the cell holder, and OD_{600} was measured after equilibration. Choice of wavelength for the measurement was dictated by knowledge of the extinction coefficient of bound I_2 at 600 nm as determined previously by Cesaro;[51] the wavelength of maximum absorbance varied from 560 to 580 nm with increasing concentration of I_2. All samples of CMA were studied at low α to minimize electrostatic repulsions between carboxylate groups and between carboxylates and the negatively charged bound species, thereby to ensure maximum iodine binding capacity. Measurements were carried out with HCMA prepared by cation exchange of CMA or with nonexchanged CMA at pH values below 2.5. Polymer concentrations were adjusted to values ranging from $C_{ps} = 3.6 \times 10^{-3}$ g dl^{-1} for CMA-I to $C_{ps} = 2.2 \times 10^{-2}$ g dl^{-1} for CMA-V in order to obtain convenient OD values. These concentrations were determined either by dry weight analysis of NaCMA stock solutions in pure water prior to addition of KI, or, for samples of higher DS, by carboxylate group titration as described above.

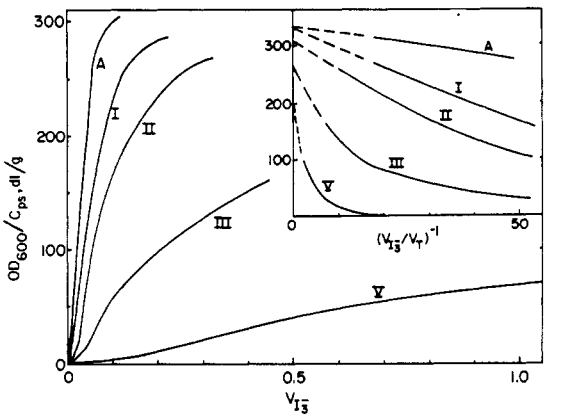

Figure 1. Plots vs. volume of added iodine of the optical density at 600 nm of iodine complexes of amylose and CMA at low α divided by polymer concentration for use in determinations of iodine binding capacities as described in the text.

For titrations of type (2) the pH of a solution of CMA-III ($C_{ps} \simeq$ 0.02 g dl^{-1}) in aqueous 0.01 M KI containing 1×10^{-4} M I_2 was adjusted to a desired value in the range 2.4–4.0 by the addition of 0.2 N HCl or NaOH as required. An aliquot was withdrawn to the spectrophotometer cell, the spectrum was measured in the region 400–800 nm, and the sample was returned to the bulk solution for repetition of the process at another pH. A record was kept of the volumes of acid and base added to the original bulk solution, so that measured optical densities of the CMA–I_2/I^- complex could be corrected for polymer dilution. The visible spectra of CMA-V solutions were obtained as a function of pH following similar procedures using concentrations $C_{ps} \simeq 0.05$ g dl^{-1}, [KI] = 0.05 M, and $[I]_2 = 2 \times 10^{-4}$ M. These concentrations lead to convenient OD values. The C_{ps} values were determined by titrations of portions of CMA stock solutions in pure water prior to volumetric dilution with solutions of KI and I_2. Spectrophotometric titration with 0.2 N HCl of appropriate blank solutions containing only aqueous KI and I_2 disclosed no pH dependence of $[I_3^-]$ or $[I_2]$ in the pH range investigated; these concentrations were monitored by measuring the OD at 353 and 288 nm.[52]

Results

Spectrophotometric Determination of Iodine Binding. Spectrophotometric CMA–I_2/I^- binding isotherms are reported in Figure 1 as OD_{600}/C_{ps} vs. ml of titrant $V_{I_3^-}$ for CMA samples of varying DS and at low α; a similarly constructed amylose–I_2/I^- binding isotherm reported by Cronan and Schneider[53] at 20°C and in 0.01 M ki is shown for comparison. To measure the iodine binding capacity of am-

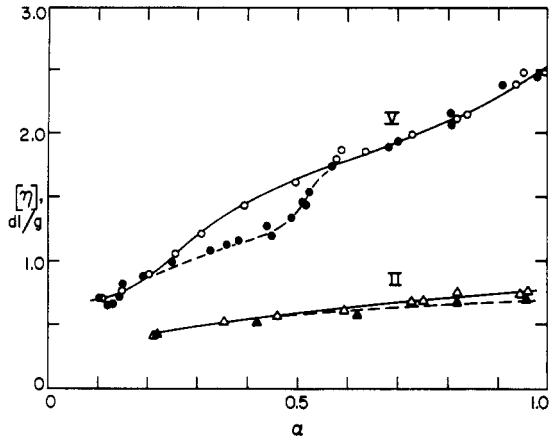

Figure 2. Plots of the intrinsic viscosity of CMA-II and -V vs. α in 0.05 M NaCl in the presence (filled symbols) and absence (open symbols) of 6% BuOH. The solid curve is drawn to conform to the open symbols; the dashed curve follows the filled symbols in that range of α for which the solid curve does not adequately fit these data.

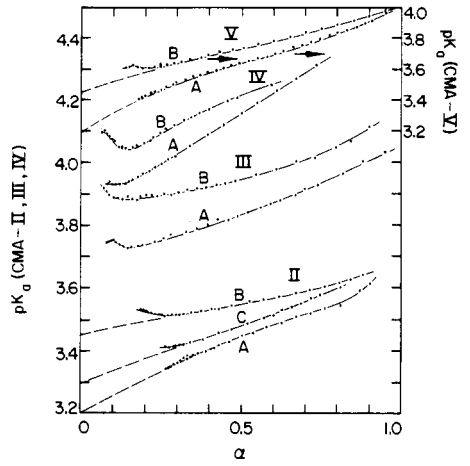

Figure 3. Plots of pK_a (left ordinate) vs. α for CMA-II at C_{ps} = 0.8 g dl^{-1} in (A) 0.05 M NaCl, (B) 0.05 M NaCl with 6% BuOH, (C) 0.05 M KI with 3.6 × 10^{-4} M I_2; for CMA-III at C_{ps} = 2.0 g dl^{-1} in (A) pure water, (B) water with 8% BuOH; for CMA-IV at C_{ps} = 1.0 g dl^{-1} in (A) pure water, (B) water with 8% BuOH. Plot of pK_a (right ordinate) vs. α for CMA-V at C_{ps} = 0.5 g dl^{-1} in (A) 0.05 M NaCl, (B) 0.05 M NaCl with 6% BuOH.

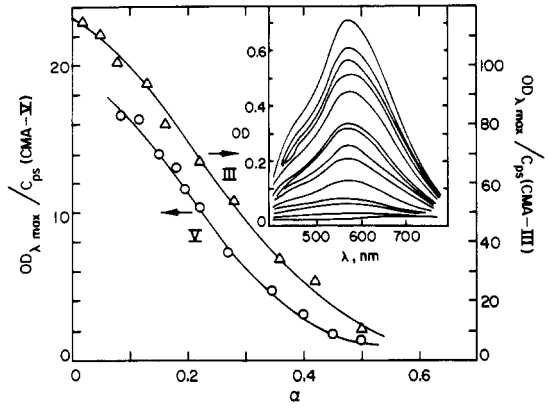

Figure 4. Plots vs. α of the optical density at the absorption maxima of the iodine complexes (shown in inset for CMA-V) divided by polymer concentration for CMA-III (right ordinate) and CMA-V (left ordinate). Experimental conditions are described in the text.

ylose and CMA the optical density normalized by the polymer concentration OD_{600}/C_{ps} was evaluated at infinite iodine concentration by plotting against inverse total iodine concentration $(V_{I_3^-}/V_T)^{-1}$ and extrapolating to zero as shown in the inset of Figure 1; here V_T is the total volume of the solution. The extrapolation becomes tenuous for the samples of higher DS leading to an uncertainty of ±15–20% in the iodine binding capacity reported in Table I for CMA-V. The binding capacities in Table I were obtained by dividing the extrapolated values of OD_{600}/C_{ps} by the extinction coefficient of bound iodine.[51] This yields the ratio of concentrations of bound iodine and total polymer at saturation, and this may be converted to the customary units reported in Table I, i.e., g of I_2/100 g of amylose. Binding capacity values reported in Table I for CMA have all been "normalized" to a per 100 g of amylose basis by multiplying the binding capacity of the derivative by the factor M_u/M_r.

Intrinsic Viscosities. Intrinsic viscosities of CMA-II and -V in aqueous 0.05 M NaCl at 25°C in the presence and absence of 6% BuOH are reported as a function of α in Figure 2. The values of α were determined from the measured pH in conjunction with the titration curve of the appropriate sample of CMA in the given solvent as described above. Titration data for CMA-V extended only to $\alpha \simeq 0.12$ and 0.18 (pH 2.86 and 2.73) in the presence and absence of BuOH, respectively; the corresponding lower limits of α for CMA-II were ca. 0.20 and 0.25 (pH 2.89 and 2.86). Hence, it was necessary to obtain values of α for some of the viscosity experiments conducted near the lower limits of α shown in Figure 2 by extrapolation of the titration data into the region of α below the experimental range as explained below. Scatter in the data of Figure 2 is due principally to the ±2% uncertainty in $[\eta]$ and the uncertainty of ±0.02 in α reported above. Although considerable scatter occurs in the data at low α in the presence of BuOH, the indicated convergence of the viscometric titration curves for CMA-V at low values of α appears to be real, and this observation receives support from the behavior of CMA-II. For the latter polymer the linear charge density ρ, defined by $\rho = \alpha DS$, does not exceed 0.12 throughout the experimental range of α; this charge density is achieved by CMA-V for $\alpha = 0.22$. Thus, for neither CMA sample is there a discernable influence of 6% BuOH on $[\eta]$ for $\rho < 0.12$. The intrinsic viscosities of CMA-II and -V at low α were used in conjunction with the Flory–Fox equation[25] and the viscosity average degrees of polymerization given in Table I to estimate the values of $\langle r^2\rangle/xl^2$ are shown in Table I. Here $\langle r^2\rangle$ is the mean-square end-to-end distance, x is the degree of polymerization, and l is the distance (assumed to be fixed at 4.25 Å) between the oxygen atoms of consecutive glycosidic linkages.

Potentiometric Proton Titrations. Representative titration data are shown in Figure 3 as pK_a vs. α for CMA-II, -III, -IV, and -V at varying ionic strengths and polymer concentrations, in the presence and absence of BuOH and I_2/I^-. The logarithmic apparent dissociation constant pK_a is defined by $pK_a = pH + \log[(1 - \alpha)/\alpha]$.[54,55] Rather high polymer concentrations were employed in these titrations in order to obtain data at low α, since self-ionization of the protonated form HCMA leads to high initial degrees of dissociation at low equivalent concentrations of polyacid. The lengthy extrapolations to the logarithmic intrinsic dissociation constant pK_0 drawn for samples II and V in 0.05 M salt are supported by the agreement of pK_0 values for these two polymers in a given solvent medium.[46] Values of α, required to construct Figure 2 and corresponding to pH values below the experimental titration range, were obtained from the extrapolations of curves labeled A for CMA-II and -V in 0.05 M NaCl in the absence of BuOH. Titration data obtained in the presence of BuOH extended

to sufficiently low α for CMA-II and required an extrapolation of only 0.02 α units (not shown) for CMA-V. It should be noted that an extrapolation of the pK_a vs. α curve which follows a path lower than the actual curve would lead to an overestimate of α, whereas too high an extrapolation would cause the estimate of α to be too low.

Spectrophotometric Titrations with Acid and Base. The visible absorption spectrum of the I_2/I^- complex of CMA-V at pH values in the range $2.4 \leq pH \leq 4.0$ is shown in the inset in Figure 4. The highest curve corresponds to pH 2.4, the lowest to pH 4.0. The OD values at the maxima of these curves, divided by the respective polymer concentrations, are presented as a function of α in Figure 4. The value of α at each pH was determined from the titration curve of CMA-V in 0.05 *M* NaCl, since titration data were found to be nearly identical in aqueous NaCl and aqueous KI solutions of equal ionic strength. Similar results for the I_2/I^- complex of CMA-III are also shown in Figure 4.

Discussion

Choice of Polymer Samples. Iodine binding capacities reported in Table I disclose that the capacity of CMA at low α to bind iodine falls below that of amylose for DS values exceeding about 0.3; the iodine binding capacity of CMA-V (DS = 0.55) is approximately 85% that of amylose whereas those of CMA-II (DS = 0.12) and amylose are identical. It may be presumed that the iodine binding capacity of CMA in comparison with that of amylose reflects the steric and/or electrostatic perturbation of the amylose V helix due to carboxymethyl substitution. It is apparent from the binding isotherms in Figure 1 that even for CMA of low DS and α the tenacity of iodine binding is reduced by carboxymethylation, i.e., the rate of approach to saturation with increasing iodine concentration is diminished in comparison with the isotherm for amylose. It is not possible to deduce from the present data the extent to which this reduction in binding stability results from steric effects as opposed to electrostatic repulsion between negatively charged carboxylate groups and the negatively charged bound species.

The suitability of CMA as a model for unsubstituted amylose in the absence of helicogenic agents has been discussed elsewhere.[4,6,25] The virtual identity of the aqueous characteristic ratio C_∞ for CMA of DS = 0.3 with that of amylose in neutral aqueous solvents suggests strongly an identity of chain configuration for amylose and CMA of DS $\leq$ 0.3 in aqueous solution in the absence of helix-inducing compounds; for CMA with DS > 0.3 C_∞ is known to exceed that of amylose in aqueous solution.[4] Intrinsic viscosity measurements conducted in the present study for CMA-II and -V in 0.05 *M* aqueous NaCl at low α yield the values of $\langle r^2 \rangle / xl^2$ shown in Table I. Inasmuch as this medium is apparently a Θ solvent for amylose, these values for slightly charged CMA obtained at relatively high ionic strength should correspond closely to Θ conditions and thus be comparable with the characteristic ratio of amylose itself. It is evident that for CMA-II the chain dimensions in this medium closely approximate those of unperturbed amylose, whereas CMA-V possesses a somewhat expanded coil. Choice of CMA-II and -V for the present investigation was dictated by the desire to study (1) a polymer, i.e., CMA-II, closely resembling amylose in its behavior both in the presence and absence of helicogenic agents, and (2) a polymer, i.e., CMA-V, capable of achieving a linear charge density ρ sufficient to produce pronounced polyelectrolyte behavior.

Analysis of Intrinsic Viscosity Results. Both CMA-II and -V show the expected increase in $[\eta]$ with increasing α in Figure 2. For the latter polymer the increase amounts to a factor of 3.6 over the range $0.1 \leq \alpha \leq 1.0$, whereas for CMA-II the factor is only 1.7 in consequence of its significantly lower charge density at high α. Above $\alpha = 0.6$ there is no discernable effect of 6% BuOH on $[\eta]$ for CMA-V. This observation may be presumed to arise from the expanded character of the polyion in the region $0.6 \leq \alpha \leq 1.0$ in which segments of the polymer are prevented by electrostatic repulsive forces from adopting a helical conformation, even in the presence of 6% BuOH. It is perhaps worth noting at this point that for CMA-V in aqueous 0.05 *M* NaCl the parameter ξ discussed by Manning[56,57] does not exceed the critical value of unity for any value of α; when 6% BuOH is present, $\xi \geq 1$ only for $\alpha \geq 0.9$. In no event does ξ exceed unity for CMA-II. Consequently, phenomena observed in the present study are presumably not subject to interpretation in terms of changes in counterion mobility.

In the region $0.2 < \alpha < 0.6$ the presence of 6% BuOH depresses $[\eta]$ for CMA-V by as much as 20%, presumably because partially helical polymer molecules stabilized by the presence of BuOH cannot expand under the influence of a given linear charge density as much as can polymer molecules in which regions of compact helical structure are not so stabilized. For $\alpha \leq 0.2$, corresponding to $\rho \leq 0.11$, there is again no detectable effect of 6% BuOH on $[\eta]$ for CMA-V. This behavior is consistent, as noted above, with the observation that 6% BuOH has no significant influence on $[\eta]$ for CMA-II in the same range of ρ, i.e., $\alpha \leq 0.95$.

The absence of any influence of 6% BuOH on $[\eta]$ at low charge density is curious in view of the pronounced reduction in $[\eta]$ produced by this substance in the range $0.11 < \rho < 0.33$, i.e., $0.2 < \alpha < 0.6$, for CMA-V. It is reasonable to anticipate that the contraction in chain dimensions which occurs upon stabilization of regions of helicity in an expanded polyion coil will be larger the greater the extent of polyion expansion. As noted previously, this is apparently what one observes in comparing the effect of BuOH on the reduced specific viscosity of unsubstituted amylose in neutral aqueous solution and in 0.01 *M* KOH in which the molecule has polyelectrolyte character. In the latter medium the reduced specific viscosity is diminished in the presence of 6% BuOH by some 42% but by only 26% in the former.[7] It is not, however, evident a priori what the effect will be on the dimensions of a neutral, coiling polymer chain from induction of helical sequences incorporating even the major fraction of the monomer units; only for development of very high degrees of helicity can one confidently predict that the chain dimensions will be expanded relative to the random coil. In pursuit of an understanding of the CMA–H_2O–BuOH system it is useful to compare the α dependence of $[\eta]$ for CMA with that observed for other polyelectrolytes. Such comparisons, in conjunction with similar comparisons of potentiometric proton titration data, can provide insight into the possible existence of helical order in the aqueous CMA chain in the presence and absence of BuOH.

One may consider first whether the dependence of $[\eta]$ on α for CMA-V corresponds simply to polyelectrolyte expansion of an essentially random configuration, or, rather, involves the disruption of ordered regions in the polymer chain, presumably similar to those present in one of the known crystalline forms. Experimental data believed to correspond to the former type of process are presented in Figure 5a. The dependence of $[\eta]$ on α for a number of randomly coiled polycarboxylic acids is observed to be remarkably uniform when plotted on semilogarithmic coordinates.[44b] Both polyacrylic acid (PAA) and ethyl vinyl ether–maleic acid copolymer (EVE-MA) behave as typical weak polyacids, and their log $[\eta]$ vs. α curves are similar. On the

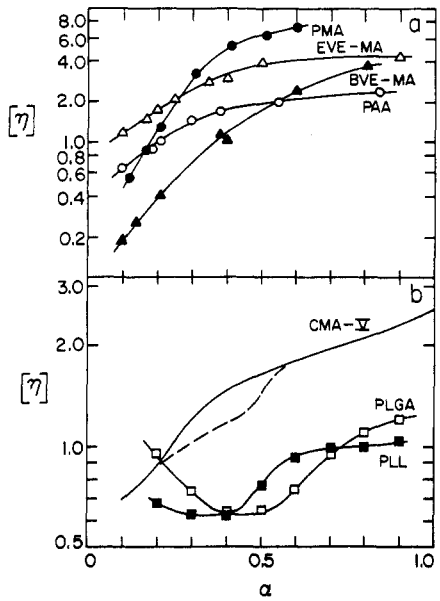

Figure 5. (a) Plots of log $[\eta]$ vs. α for PAA in 0.10 *M* NaCl (open circles),[43a] PMA in 0.10 *M* NaBr (filled circles),[43b] EVE-MA in 0.04 *M* NaCl (open triangles),[44a] and BVE-MA in 0.04 *M* NaCl (filled triangles).[44a] (b) Plots of log $[\eta]$ vs. α for PLGA in 0.20 *M* NaCl (open squares),[47] PLL in 0.20 *M* NaCl (filled squares),[48] and CMA-V in 0.05 *M* NaCl (solid curve) and in 0.05 *M* NaCl with 6% BuOH (dashed curve).

other hand PMA and BVE-MA assume highly compact "hypercoiled" conformations at low α. (Note that for the poly(diprotic acids) EVE-MA and BVE-MA the polyion is only half dissociated at $\alpha = 1$.[44]) The hypercoiled state is apparently stabilized by hydrophobic interactions among the backbone substituents.[43,44] Although the fractional increase in $[\eta]$ with increasing α is greater for PMA and BVE-MA at low α, the general shape of log $[\eta]$ vs. α is the same for all four polyacids, and the curves in each case appear to be linear below $\alpha = 0.3$. It seems probable that these similarities exist in spite of the hypercoiling phenomenon in PMA and BVE-MA, because the mutual orientation of chain segments remains essentially random even in the hypercoiled state. In contrast to the behavior just described curves of log $[\eta]$ vs. α for polymers known to undergo charge-induced transitions from helix to coil display a pronounced minimum in $[\eta]$ with increasing charge density. Curves of this type are shown in Figure 5b for PLGA and PLL. (For the latter polymer α is the degree of ionization rather than the degree of dissociation.) The higher values of $[\eta]$ which occur at low α for these polymers are associated with the presence of extensive rigid helical structure in the molecule, whereas polyelectrolyte expansion of the randomly coiling polymer leads to the increase in $[\eta]$ at high α.

The present intrinsic viscosity data for CMA-V are plotted in corresponding fashion in Figure 5b for comparison with the other curves. The curves shown in Figure 5b correspond to the smoothed curves drawn through the experimental data in Figure 2. Even in the presence of 6% BuOH CMA-V fails to display the minimum exhibited by PLGA and PLL. Moreover, the dependence of log $[\eta]$ on α when BuOH is present is also clearly to be differentiated from the curves for the nonhelical polycarboxylic acids, both those which display cooperative transitions to the hypercoiled state and those which do not. There is an apparent inflection point in the log $[\eta]$ vs. α curve for CMA-V in the absence of BuOH near $\alpha = 0.2$. The reality of this change in curvature receives some support from two other investigations of the polyelectrolyte behavior of CMA[18,19] which suggest that the dependence of $[\eta]$ on pH vanishes at low pH. In the absence of the corresponding titration curve it is, however, impossible to convert the data of these authors to a plot of log $[\eta]$ vs. α for direct comparison with the present results. As noted, values of $\alpha < 0.18$ were evaluated for intrinsic viscosity experiments with CMA-V in systems lacking BuOH by using the extrapolated titration curve labeled A in Figure 3. Given the observed upturn at $\alpha \simeq 0.1$ of the other titration curves for solutions without BuOH, the extrapolation may be lower than the actual curve. Values of α calculated from a higher titration curve would be smaller than those shown in Figure 2, and the apparent inflection in log $[\eta]$ vs. α would become more pronounced. Consequently, we believe that this inflection is real and that even when BuOH is absent, log $[\eta]$ vs. α for CMA-V does not mimic the behavior of the nonhelical polycarboxylic acids.

It is therefore interesting to consider whether the CMA-V curve can be viewed as similar to those of PLGA and PLL in the region of α where α exceeds its value at the minimum in the viscosity curves for the latter polymers. If we imagine that the curve of log $[\eta]$ vs. α for CMA-V in the absence of BuOH corresponds in the range $0.1 < \alpha < 0.6$ to the electrostatic disruption of helical, *or otherwise compact*, sequences, then it would be expected that introduction of a helicogenic agent would shift this portion of the curve to higher values of α by enhancing the stability of compact helical sequences. Such a shift is indeed observed upon addition of 6% BuOH to solutions of CMA-V in aqueous 0.05 *M* NaCl as can be seen in Figure 5b. The observed convergence of the intrinsic viscosity curves for CMA-V with and without BuOH for $\alpha \leq 0.2$ is consistent with this interpretation of the two curves in the range $0.2 < \alpha < 0.6$ only if the incorporation of BuOH-stabilized helical structure produces no change in the dimensions of the *uncharged* CMA chain. The possibility that at still lower charge densities than were achieved with CMA-V the intrinsic viscosity in the presence of BuOH exceeds that in the absence of BuOH can be dismissed in view of the superposition of the two viscosity curves for CMA-II shown in Figure 2 at values of α which correspond to charge densities as low as one charged group per 40 glucose residues. (Note that the intrinsic viscosity curve for CMA-II can justifiably be viewed as an extension of the curve for CMA-V to lower charge densities, inasmuch as the measured intrinsic viscosities of the two samples are similar at a given value of ρ. Compare, for example, the intrinsic viscosities in Figure 2 of CMA-V at $\alpha = 0.2$ and CMA-II of $\alpha = 0.95$ where for both polymers $\rho = 0.11$.) The intrinsic viscosity data then are consistent with a model for CMA-V which envisages stabilization by 6% BuOH of compact, presumably helical, residue sequences in the range $0.0 \leq \alpha < 0.6$. For $0.2 < \alpha < 0.6$ the observed effect of BuOH is to inhibit the polyelectrolyte expansion of the chain; the compact chain sequences are presumed to be unstable for $\alpha \geq 0.6$, where 6% BuOH has no effect at all on the chain dimensions. When $\alpha \leq 0.2$, incorporation of BuOH-stabilized compact sequences again produces no measurable change in the chain dimensions. Information concerning the possible existence of locally compact residue sequences postulated in this model to occur at low α may be obtained from analysis of the potentiometric titration data.

Analysis of Potentiometric Titration Results. The logarithmic apparent dissociation constant pK_a, plotted as a function of α in Figure 3, can be regarded as a measure of the ease of proton removal from the polyion at a given degree of dissociation.[54,55] For polycarboxylic acids it is anticipated that pK_a will increase with increasing volume densi-

ty of negative charge σ in the vicinity of the polyion. At low α and ρ the value of σ can normally be expected to increase with increasing ρ. At higher ρ the rate of increase of σ may be diminished by aggregation of counterions in the domain of the polyion.[56,57] In unusual cases, characterized by cooperative transformation from a compact to an expanded chain configuration, σ may actually decrease with increasing ρ such that pK_a exhibits a negative slope with α over some range of α. Such titration behavior is in fact usually taken as diagnostic of a cooperative charge-induced conformation change in the macromolecule.[46,55] Extrapolation of pK_a to vanishing charge density yields pK_0, the logarithmic intrinsic or microscopic dissociation constant for the (identical) dissociable groups of the polymer.[46,54] Thus, plots of pK_a vs. α for BVE-MA and EVE-MA in 0.04 *M* aqueous NaCl extrapolate to a common value of pK_0, but whereas pK_a is a monotonically increasing function of α for EVE-MA, the curve for the hypercoiling BVE-MA displays a maximum and a minimum with an intervening region of negative slope.[44] Similar results are obtained for the pairs PMA-PAA[43a] and PLGA-PDLGA (poly(D,L-glutamic acid)).[46] In the presence of supporting electrolyte the value of pK_a should be sensitive to the average *local* configuration of ionizable groups in the vicinity of a given ionizable group.[56] Hence studies of pK_a as a function of α may in some cases probe features of the chain configuration different from the mean chain dimensions to which the value of $[\eta]$ is sensitive. In particular it should be sensitive to the presence of locally compact regions of the polycarboxylic acid chain,[55] e.g., helical residue sequences in polypeptides like PLGA or hydrophobic micelles in polyacids like BVE-MA.

In the present case the value of pK_0 appears to be independent of DS as anticipated, inasmuch as the pK_a data for CMA-II and -V extrapolate to the same intercept in a given solvent system. In agreement with titration data for other polycarboxylic acids[46,54,55,58,59] the value of pK_0 is found to be strongly dependent on ionic strength, e.g., pK_0 for CMA-III in 0, 0.05, and 0.78 *M* aqueous NaCl was found to be 3.9, 3.5, and 3.2, respectively; this accounts for the vertical displacement of the data for CMA-II and -III in Figure 3, obtained at NaCl concentrations of 0.05 *M* and 0, respectively. In the absence of salt, titration curves become strongly dependent on polymer concentration, since the effective ionic strength in the environment of a polyion is essentially determined by the counterions of its neighbors, hence, the vertical separation of data for CMA-III and -IV. The observed dependences of pK_a and pK_0 on salt and polymer concentration conform qualitatively to theoretical expectations.[54,57] Higher values of pK_0 are obtained for a given CMA sample and salt concentration in the presence of BuOH; the method used to standardize the pH meter precludes any meaningful interpretation of this observation.[50]

Many of the curves in Figure 3 exhibit a reduction in positive slope and, indeed, in some cases a region of negative slope at low α. The curves shown are representative of numerous titrations, and the upturn at low α is reproducible. We are convinced that it is not an artifact resulting from inaccurate determinations of α, which can introduce errors in pK_a at the extremes of the range of α. Hence, we believe that the titration behavior at low α corresponds to a process in which the average separation of carboxyl groups changes sufficiently rapidly with α to cause σ and ρ to vary in the opposite sense. As is the case with certain ionic polypeptides, the rapid change in average separation of carboxyl groups could arise either from an intermolecular or an intramolecular process,[60] i.e., it could involve either aggregation or a molecular conformation change. The former

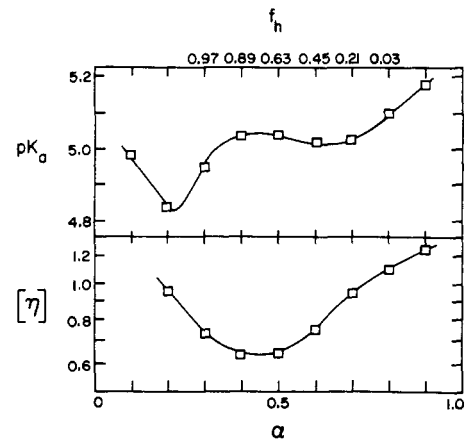

Figure 6. Plots of pK_a and log $[\eta]$ vs. α (lower abscissa) and f_h (upper abscissa) for PLGA in 0.20 *M* NaCl.[47]

phenomenon must certainly be considered at the relatively high polymer concentrations which were employed here to obtain data at low values of α. We have, however, observed no enhancement of the upturn at low α upon increasing polymer concentration (compare curves for CMA-III and -IV) or increasing ionic strength (compare curves for CMA-II and -III), both of which would be expected to facilitate intermolecular aggregation if it were occurring. Patel and Patel[19] have reported a similar upturn in pK_a at low α for CMA of DS = 0.8. As polymer concentration was increased in their studies the upward deviation did not increase and, indeed, appeared to diminish somewhat. Comparison of the present data for titrations with and without BuOH shows that the upturn is apparently enhanced by this cosolvent. There is some indication of a similar effect upon addition of I_2/I^- in the data for CMA-II, and Rao and Foster[18] previously observed such an effect of iodine on CMA. Although the effect could be due to intermolecular aggregation facilitated by the helicogenic species, we believe the evidence favors induction of an intramolecular conformation change. Titrations of CMA-III and -IV were conducted at high C_{ps} to allow the data to extend below $\alpha = 0.1$ and in the absence of salt to permit use of a higher concentration of BuOH, i.e., 8%. Only under these conditions of very low α and no added salt do we observe an upturn in pK_a in the absence of helicogenic cosolvent. This upturn may reflect the occurrence of compact sequences in CMA at low α even when helicogenic agent is absent, and this is consistent with the apparent inflection point in the log $[\eta]$ vs. α data for CMA-V in the absence of BuOH. It may also be significant that the effect of BuOH on the titration curve appears to be most pronounced for these titrations which were conducted at higher BuOH concentration and carried to very low α. It is not clear, however, that disruptive electrostatic forces are smaller in these cases than for CMA-II, or even CMA-V, at somewhat higher α but in the presence of 0.05 *M* NaCl.

Comparison of Viscosity and Potentiometric Titration Results. The relationship between the intrinsic viscosity and potentiometric titration results for CMA may be considered in light of Figure 6 where pK_a and log $[\eta]$ are plotted as functions of α and f_h (fraction of residues in the helical state) for PLGA.[47] The plot of pK_a for PLGA shows a maximum at $\alpha \simeq 0.45$ and a minimum at $\alpha \simeq 0.60$ and is characteristic of titration curves of polycarboxylic acids undergoing a cooperative charge-induced conformation change. The region of negative slope in the range $0.1 \simeq \alpha \simeq 0.2$, which we subsequently disregard in this discussion, is attributable to intermolecular aggregation;[60] PLGA pre-

cipitates from aqueous solution at low α. It is noteworthy that the maximum in pK_a and the minimum in log $[\eta]$ coincide at $\alpha \simeq 0.45$ where $f_h \simeq 0.75$. Thus, the region of negative slope in the plot of log $[\eta]$ vs. α corresponds to molecules possessing very high degrees of helicity ($f_h > 0.75$) for which the pK_a increases most rapidly with increasing α as a consequence of the compact character of the molecule. The value of α at the minimum of the pK_a function ($\alpha \simeq 0.60$) corresponds closely to the point at which an inflection occurs in the log $[\eta]$ curve in the region of positive slope. Under these conditions roughly one-half ($f_h \simeq 0.45$) of the PLGA residues exist in helical sequences averaging approximately 20 residues in length.[47]

If we may take the potentiometric and viscometric behavior of PLGA in the range $0.6 < \alpha \leq 1.0$ as a model for aqueous CMA in the presence of saturating amounts of BuOH, i.e., 6–8%, we observe that the minima in pK_a vs. α which appear in Figure 3 at low α and the positive slope of log $[\eta]$ vs. α in the same range of α for CMA-V in Figure 5b are consistent with the stabilization of helical structure possessing f_h perhaps even approaching 0.5. Similar conclusions can be drawn from a comparison of the present results with those for PLL.[48] Failure to observe for CMA in the presence of saturating BuOH a minimum in log $[\eta]$ or a maximum in pK_a in plots against α may be consequence of (1) an equilibrium situation which does not correspond to high values of f_h, (2) a weakly cooperative transition which leads to rather short helical sequences even for values of f_h approaching unity, (3) stabilization of irregular or wormlike helix which consequently does not mimic the behavior of PLGA and PLL, or (4) some combination of these factors. The apparent inflection point in the plot of log $[\eta]$ vs. α for CMA-V in the absence of BuOH is reminiscent of the behavior of the helix-forming polypeptides PLGA and PLL and suggests the occurrence of rather compact residue sequences in CMA, even in the absence of helicogenic agents, when ρ is small. This suggestion is supported by the corresponding potentiometric titration at very low α.

Analysis of Spectrophotometric Titration Data. The spectrophotometric titration data shown in Figure 4 clearly reveal the destruction of the CMA–I_2/I^- complex with increasing ρ. The inability of CMA at high α to form an I_2/I^- complex has been noted previously.[18] The destruction of complex occurs over the same range of α for the two sets of data shown in Figure 4 only because the large ρ possessed by CMA-V at a given α is compensated by a higher ionic strength. The disappearance of complexed iodine with increasing α could occur concurrently with charge-induced destruction of helical sequences. On the other hand, the bound species, although of uncertain stoichiometry,[53] clearly carries negative charge, and the reduction in binding affinity could be a consequence of simple electrostatic factors without a concomitant polymer conformation change. The absence of an influence of uncharged BuOH on the dimensions of CMA-V for $\alpha \geq 0.6$, as revealed by the present intrinsic viscosity data, cannot be explained by the latter mechanism. Although the formation of a helical CMA–I_2/I^- complex is evidently deterred by high polymeric charge density, these data do not provide evidence for or against the existence of helical sequences in the absence of I_2/I^-.

Comparison of the Behavior of Amylose and CMA in the Presence of Aqueous BuOH. The absence of any detectable effect of 6% BuOH on the intrinsic viscosity of aqueous CMA at very low charge density is apparently in conflict with the report of Banks and Greenwood that BuOH causes a diminution in the reduced specific viscosity of aqueous amylose solutions.[7] We note initially that the experiments of Banks and Greenwood were evidently not conducted by bringing the polymer solution into dialysis equilibrium with the multicomponent solvent system. This circumstance raises concern about whether Banks and Greenwood were investigating an equilibrium system. Amylose is known to precipitate spontaneously from water and aqueous BuOH solutions, the crystalline morphology of the precipitate being dependent on the concentration of BuOH.[5] In our experiments with CMA we had difficulty in reproducing intrinsic viscosity values in the presence of BuOH when the BuOH was simply pipetted directly into the aqueous polymer solution rather than being dialyzed in. Furthermore, presuming that equilibrium did obtain in their system, Banks and Greenwood report measurements of reduced specific viscosity conducted at a polymer concentration of about 0.1 g/dl rather than the intrinsic viscosity. The variation of reduced specific viscosity with polymer concentration normally shows a strong dependence on solvent composition, particularly when preferential absorption of one solvent component, e.g., BuOH, may occur.[61] In one reported instance the slope of this function was in fact observed to be negative when the multicomponent solvent system was not brought into dialysis equilibrium with the polymer solution.[44b] Hence, it is not clear that the dependence of the reduced specific viscosity of aqueous amylose on the concentration of added BuOH is a reliable indication of the dependence of the intrinsic viscosity on this parameter. Finally, we observe that the apparent discrepancy in the integrity of the helical structure formed in the presence of BuOH by amylose and CMA. Our sample CMA-II is substituted on one residue out of every eight. This frequency of substitution could conceivably diminish significantly the regularity of helical sequences stabilized by BuOH in CMA-II in comparison with helices formed in unsubstituted amylose. Data presented in Figure 1 indicate that I_2/I^- is bound less tenaciously to CMA than to amylose. On the other hand, Table I shows that the saturation binding capacity of CMA for I_2/I^- is the same for CMA and amylose providing DS $< \sim 0.3$ for the CMA. Our theoretical calculations[62] on the dimensions of amylosic chains in the helix–coil transition region suggest, however, that qualitatively different effects upon the chain dimensions from helicogenic agents are not to be anticipated for amylosic chains containing sequences of *regular* V or B helix as compared to chains containing helical sequences with less structural integrity.

Interpretation and Conclusions

Evidence adduced in the present investigation supports a model for aqueous CMA in the presence of saturating BuOH which envisages stabilization of helical sequences in the polymer chain at sufficiently low polymeric charge density. The helical structure is presumably similar to that of crystalline V-amylose in which BuOH molecules reside in an annular cavity within the helix. At high linear charge density the polymer is prevented from forming helical sequences by strong intramolecular electrostatic interactions. Even at low charge density the development of helical character in this medium is not sufficient to produce the dramatic phenomena in graphs of log $[\eta]$ and K_a vs. α characteristic of polymers, e.g., PLGA, known to undergo charged-induced transformations from rigid helix to random coil. The data suggest instead the formation at low α and in the presence of saturating BuOH of a chain configuration possessing significant amounts of helical structure, but one which fails to mimic completely the behavior of PLGA and PLL. In terms of a model which allows for the occurrence in a given CMA chain of glucose residues in both helical and coil states the incomplete correspondence of CMA in this medium with the behavior of the ionic poly-

peptides might be imagined to arise because the maximum attainable equilibrium value of f_h remains small or because development of long helical sequences does not occur even for large f_h due to weak cooperativity of the transition. If, on the other hand, the wormlike chain model is adopted, then the lack of correspondence may be attributable to the flexibility of the wormlike chain in comparison with the more rigid polypeptide α helix. Finally, it must be acknowledged that the regularity of helical sequences in CMA may be significantly impaired by carboxymethyl substitution of occasional hydroxyl groups leading to behavior at low α substantially different from that of the ionic polypeptides. Indeed, any of the above cited factors could combine to render the dependence of log $[\eta]$ and pK_a on α for CMA different from that for PGLA and PLL.

When data gathered in the absence of BuOH are examined, the inference may be drawn from the apparent inflection point in log $[\eta]$ vs. α and the minimum in pK_a vs. α at very low α that CMA in the absence of helicogenic agent may adopt a chain configuration at low charge density possessing helical residue sequences. Neither in the presence nor absence of BuOH does the dependence of log $[\eta]$ on α appear to resemble that for the nonhelical weak polyacids, whether they undergo simple polyelectrolyte expansion with increasing α as do PAA and EVE-MA or whether, like PMA and BVE-MA, they display cooperative transformations from a hypercoiled configuration. It is consequently reasonable to conclude that aqueous CMA at low charge density is also helical or partially helical in the absence as well as in the presence of helicogenic agent. The appeal of this inference is especially strong in view of the identity of $[\eta]$ in the charge density range $0.0 \leq \rho \leq 0.11$ for CMA in the presence and absence of BuOH. Thus, it may be imagined that partially helical or wormlike aqueous CMA at low ρ, unperturbed by volume exclusion or charge expansion, interacts *through its existing helical structure* with added BuOH and consequently suffers no change in chain dimensions or intrinsic viscosity. Upon increases in α and ρ CMA chains possessing BuOH-stabilized helical sequences are more resistant to polyelectrolyte expansion than are CMA chains in the absence of BuOH until for $\rho \geq 0.33$ the BuOH-stabilized helical sequences are disrupted by electrostatic interactions, and the intrinsic viscosities in the presence and absence of BuOH again become identical. We wish to point out in what follows that a random coil chain model for slightly charged aqueous CMA in the absence of helicogenic agent is also consistent with the experimental observations reported here.

A theoretical treatment of the configurational statistics of amylosic chains[5,6] has been described above. The treatment produced excellent agreement with experimental observations[4] of the chain length and temperature dependences of the characteristic ratio C_x of two amylose derivatives in aqueous solution in the absence of helicogenic substances, and these derivatives, which included CMA, were shown to be suitable models for the unsubstituted amylose chain in the solvent system in question. The seemingly successful statistical mechanical treatment took into account interactions among nearest neighbor glucose residues in the chain sequence; no interactions of longer range in the chain sequence and of the sort expected to characterize regular helical conformations, e.g., hydrogen bonds, were invoked in recognition of the hydrogen-bonding character of the aqueous solvent medium.

The theoretical methods employed permit a rather detailed description of the chain configuration, because the probability for any conformational state of a skeletal unit or of a sequence of skeletal units may be calculated.[35,36] Using these procedures Brant and Dimpfl[6] showed that the probability for occurrence of sequences of residues recognizable as several turns of left-handed helix was vanishingly small; the probability of similar right-handed helical sequences was still more minute. In particular, *if their model is correct*, the probability is 0.21 that an arbitrary sequence of six residues will exist at any one time in conformations possessing left helical chirality such that this sequence might be construed as one turn of a six-fold left-handed helix. This model then describes a chain with some propensity for adopting residue conformations in short sequences reminiscent of loops of left-handed helix, but this tendency seldom persists over more than a few residues and rarely over as many as two left-handed helical turns (probability $\simeq 0.04$). In the small fraction of residue sequences possessing persistent left chirality the nascent helix is disordered and entirely lacking in the regularity and helical integrity commonly associated with the term "helix." We believe it is misleading to view this chain as a disrupted, deformed, or flexible (wormlike) helix, and prefer to describe it as a random or statistical coil as a way of distinguishing it from these other generalizations which tend to focus attention on a presumed residual regularity in the backbone conformation. It should be noted, however, that many residue sequences in this random coil may occur at any given time in rather compact local conformations owing to the characteristics of the "mean residue conformation" which, when repeated in sequence, tends to generate helical sequences of very low pitch.[6]

In terms of this description of the aqueous amylosic chain in the absence of helicogenic agents the present results may be understood as follows. The propensity of aqueous amylosic chains to adopt residue sequences which approximate helical loops of very low pitch and with variable integrity and chirality contributes to the formation of many regions of locally compact structure which apparently cause the chain to differ in its polyion expansion behavior from the simple weak polyelectrolytes such as PAA and EVE-MA. Likewise the dependence of log $[\eta]$ on α for aqueous CMA in the absence of BuOH appears to be different from the behavior of the hypercoiling hydrophobic polyacids PMA and BVE-MA and seems instead to resemble more closely that of the ionic polypeptides PLGA and PLL which adopt a regular helical conformation at low charge density. When BuOH is present, however, plots of log $[\eta]$ vs. α resemble neither the plots for PLGA and PLL nor those for BVE-MA and PMA and suggest that the CM chain configuration in this medium may be qualitatively different from those of the polypeptides and the hydrophobic polyacids at low α. Whereas BVE-MA and PMA adopt a hypercoiled but essentially random configuration at low α while PLGA and PLL are highly ordered, we may attribute the behavior of CMA in BuOH-saturated aqueous solution to the existence for $\rho < 0.33$ of BuOH-stabilized helical sequences which lack, perhaps in some combination, rigidity, regularity, or length and which resemble neither the long and quite rigid regular helices of the polypeptides nor the disordered micellar structures of the hypercoiling polyacids. At sufficiently high charge density these BuOH-stabilized helical sequences are destroyed by electrostatic effects; in the absence of BuOH even at low charge density the CMA chain is seen in this model to lack significant structure recognizable as sequences of regular or flexible helix.

An explanation of the absence of any effect of saturating BuOH on the intrinsic viscosity of aqueous CMA for $\rho \leq 0.11$ might seem to present the greatest difficulties for the random coil chain model, given the evident effect of this helicogenic agent at slightly higher ρ. Preliminary calculations[42] indicate, however, that the initial incorporation of

helical sequences into random coil amylose can be expected to have rather different effects depending upon the helix chirality and the cooperativity of the coil to helix transition; in some cases the chain dimensions decrease and in others they arise or remain unchanged in the range 0–50% helical residues. It was shown that the observed absence of any influence of the helicogenic cosolvent on the chain dimensions of aqueous CMA at small values of ρ is consistent with induction by added BuOH of up to 50% of a left-handed V helix of modest cooperativity.[42] A more extensive theoretical investigation of the several models for aqueous amylosic chains discussed here is in progress and will be published in due course.[62]

It is possible that other cosolvents may be more potent than BuOH in their effect upon the configuration of CMA and thus lead to development of more extensive and regular helical sequences. We are considering several other compounds in this light with particular attention to the mode of action of helicogenic agents. There is good evidence, for example, that I_2/I^- interacts with amylose through a specific binding reaction characterized by a large formation constant and significant cooperativity,[53] but it is by no means clear that other helicogenic species interact by a similar mechanism. It is in fact conceivable that the effect of saturating BuOH on the dimensions of aqueous CMA is not a reflection of changes in degree or integrity of helical structure but rather a more generalized effect of solvation of the polymer coil.

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References and Notes

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