Second, the only interactions in the system occur when the chains intersect, always leading to an increase in energy. since mean-field theory recognizes only low-energy configurations (and not fluctuations around them), all that is required of the interaction is that it produces minima in the energy. Replacing the Edwards pseudopotential by a hard-core interaction would not alter the results of mean-field theory.

Acknowledgment. We thank R. Gaylord and M. Gottlieb for discussions and particularly for bringing the paper by Winter and Chambon to our attention. This work was partially funded by the National Science Foundation through Grant DMR87-01393. N.D.G. gratefully acknowledges receipt of an Alfred P. Sloan Foundation Fellowship and thanks Professor Seb Doniach for his hospitality at the Department of Applied Physics, Stanford University, during the final stages of the preparation of this manuscript.

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On the Aggregation and Conformational States in Aqueous Solution of a Succinoglycan Polysaccharide

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ABSTRACT: Dilute solution properties of a succinoglycan sample from *Pseudomonas* sp. (purified SHELLFLO-S) have been studied by means of light scattering, viscosity, chiroptical spectroscopy, and differential scanning calorimetry experiments. The results collected working in a wide range of NaCl concentrations clearly show that the polysaccharide undergoes a thermally induced, highly cooperative conformational transition. The nature of the transition is discussed. All evidence favors an initial partially aggregated state for succinoglycan in which single, helical chains are laterally connected via side-chain interactions. Aggregation and conformational order are disrupted on heating. On cooling, only the pristine helical state of the polysaccharidic backbones would be recovered in aqueous NaCl, at a rate strongly dependent on the salt concentration.

Introduction

From the structural point of view, many microbial polysaccharides present a high degree of regularity, which seldom is encountered in polymeric carbohydrates from other sources. Such regularity of primary structure involves the possibility that the chains may assume ordered conformations, either single or multiple helices, both in the solid state and in solution, with important outcomes for mechanical properties, in the capability to form gels, and in rheological properties.[1-6] In this context, succinoglycans, a family of structurally closely related (when not identical) exocellular polysaccharides produced by a number of different soil bacteria, appear of particular relevance.[7,8]

In this paper we wish to present a rather detailed description of the dilute solution behavior of a succinoglycan sample (purified SHELLFLO-S).[9] Data collected regard

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the dependence of the polysaccharide viscosity and chiroptical properties on added NaCl concentration and on temperature.

With the aid of information derived also from light scattering and differential scanning calorimetry experiments, a possible hypothesis is set forth on the conformational states of succinoglycan chains as a function of ionic strength and of the thermal history of the solutions.

Materials and Methods

Succinoglican (a Shell fermentation product obtained from cultures of *Pseudomonas* sp. NCIB 11592) was provided as a brown syrup containing ca. 8% of polysaccharide. After a 4-fold dilution with water and addition of NaCl (100 g/dm^3), the polymer solution was clarified by repeated filtrations ("millipore" filters, 8, 5, and 1.2 μm). Succinoglycan, precipitated by addition of excess ethanol, was redissolved in water and dialyzed exhaustively against distilled water until the absence of an excess of salt was indicated by conductivity measurements. The resulting solution was filtered (0.45 μm) to eliminate traces of residual opalescence and brought to pH 6.5, by adding dilute NaOH. The polysaccharide was recovered by freeze-drying and stored in a desiccator.

The final succinoglycan sample (N-S-1) has a water content of 14% w/W (thermogravimetric analysis) and a residual protein content of less than 1% (according to nitrogen analysis). Its equivalent weight is 726 (theoretical 754, with the repeating unit assumed to contain one succinate residue and one pyruvyl residue), as deduced from potentiometric and conductometric titrations with standard NaOH, the polysaccharide having been previously transformed into the H^+ form by ion exchange. The conductometric titrations also confirm that N-S-1 contains equimolar amounts of succinate and pyruvyl groups.

Solutions of N-S-1 were prepared by using bidistilled water; aliquots of a concentrated NaCl stock solution were added in order to reach the final, desired ionic strengths. For the light scattering experiments a stock N-S-1 solution (in 0.1 M NaCl) was dialyzed against 0.1 M NaCl in a constant-volume cell. Dilutions were performed under a laminar-flow hood by using the equilibrium dialysis "solvent" and the final solutions filtered (0.8 μm) directly into the light scattering cells.

Static light scattering measurements were carried out in the angular range 20–145° at 25 °C with the blue line (λ = 488 nm) of an Ar ion laser (Spectra Physics, Model Ar 165-06). Details on the instrument and on the elaboration of the data are given in ref 10.

Viscosity measurements were performed by using a Schott-Geraete automatic viscometer equipped with a water thermostat (±0.1 °C).

Optical activity measurements were performed with a Perkin-Elmer 241 polarimeter, using a 10-cm path length; the temperature was controlled by means of a Lauda circulating water bath (±0.1 °C).

Circular dichroism spectra were obtained with a Jasco (Model J-500A) dichrograph.

Differential scanning calorimetry experiments were carried out in the laboratory of Setaram (Paris, France) by using a Setaram-DSC. A few DSC measurements were also performed at the University of Naples (Prof. G. Barone) with an identical apparatus.

Proton NMR spectra were obtained at 85 °C, on a Varian XL-300 spectrometer.

Results and Discussion

1. Room Temperature Solution Properties of a "Native" Succinoglycan Sample. By "native" sample (N-S-1), as used in this paper, is a purified succinoglycan preparation (sodium salt) which has never undergone thermal treatments in solution and which contains pyruvyl and succinate groups in the 1:1 molar ratio (see the Experimental Section and Figure 1). Properties of N-S-1 in dilute aqueous solution at 25 °C have been studied by means of light scattering, optical activity, and viscosity measurements.

(a) Light Scattering. Relevant information on the actual state of N-S-1 chains in 0.1 M NaCl and 25 °C can

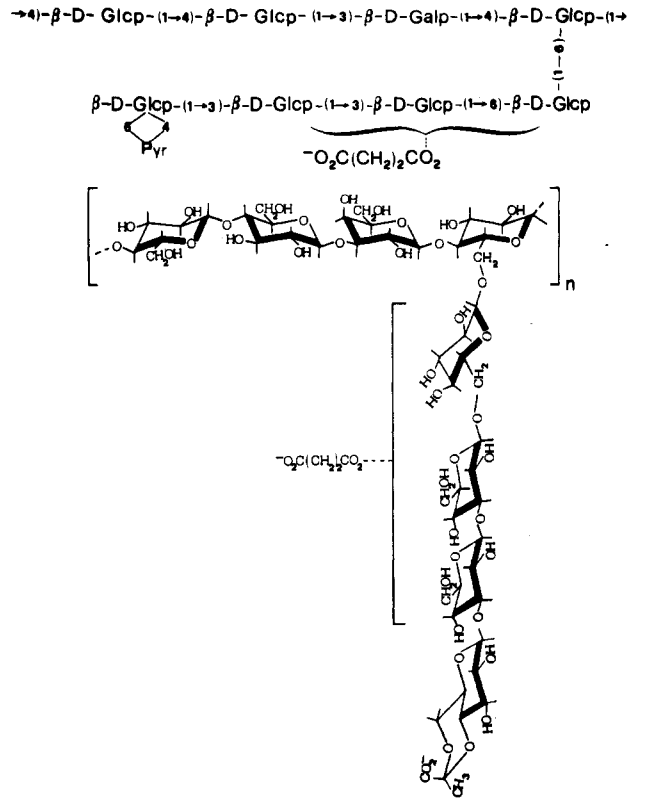

Figure 1. Structure of the repeating unit of succinoglycan. The N-S-1 sample (see text) contains 1 mol each of pyruvyl and succinate residues per repeating unit.

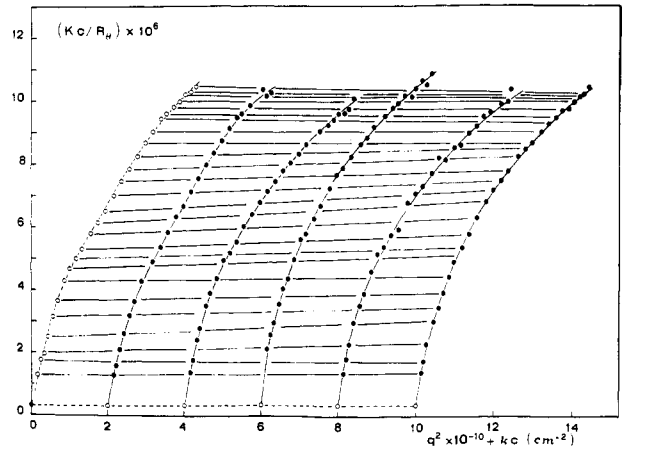

Figure 2. Zimm plot of the static light scattering measurements carried out on N-S-1 in 0.1 M NaCl at 25 °C. Polymer concentrations, c (g/dm^3): 0.2; 0.4; 0.6; 0.8; 1.0. For the refractive index increment, a value of 0.15 has been taken. (●) Experimental points, (○) extrapolated points. From the intercept on the ordinate and the initial slopes of the extrapolated curves the weight-average molecular weight, M_w; the radius of gyration, $\langle S^2\rangle_z^{1/2}$; and the second virial coefficient, A_2, have been calculated (see Table I) according to standard procedures.

be gained on the basis of our laser light scattering results. A typical Zimm plot of static light scattering data (i.e., Kc/R_θ versus $q^2 + kc$ where R_θ is the Rayleigh ratio, K the optical contrast factor, q the scattering vector $q = 4\pi\lambda \sin(\theta/2)$, k an arbitrary constant, and c the polymer concentration) is shown in Figure 2. This plot clearly shows, at large q values, a slight downward curvature which is characteristic of stiff chains.

The calculated values of molecular weight M_w, radius of gyration $\langle S^2\rangle_z^{1/2} = R_g$, and second virial coefficient A_2 are listed in Table I.[10-12] For a more detailed characterization of the solution state of N-S-1 the same light scat-

Table I
Succinoglycan (N-S-1) and Native Xanthan in Aqueous 0.1 M NaCl at 25 °C; Light Scattering Data

	N-S-1	xanthan[17]
$M_w \times 10^6$	3.3	2.9
$\langle S^2\rangle_z^{1/2}$(exptl), nm	466	290
$\langle S^2\rangle_z^{1/2}$(calcd), nm	470	298
$A_2 \times 10^4$, (cm³/mol)/g²	0	4.9
M_L(single), nm⁻¹	803	899
M_L(exptl), nm⁻¹	1000	1830
M_L(exptl)/M_L(single)	1.3	2.0
L_w, nm	3300[a]	1690[a]
N_K	12[b]	6.3[b]
l_K	287[c]	255[c]

[a] Evaluated as M_w/M_L(exptl). [b] Estimated by inserting the value of the maximum/asymptote ratio derived from the extrapolated curve shown in the Holtzer plot of Figure 3 into Figure 7 of ref 14. [c] The Kuhn segment length is calculated as $l_K = L_w/N_K$.

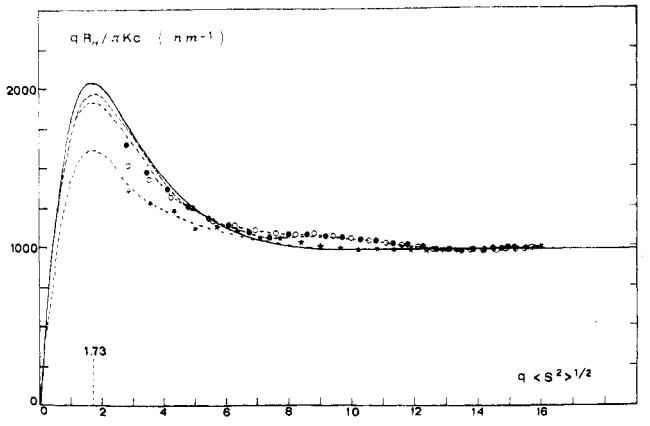

Figure 3. Holtzer plot of the light scattering data for N-S-1 in 0.1 M NaCl at 25 °C. The lowest curve (*) corresponds to a polymer concentration c = 1.0 g/dm³; the dashed curve (●) in the middle corresponds to c = 0.2; extrapolated values are (○) for c = 0. The full line has been calculated according to Koyama's theory[16] with the set of parameters given in Table I.

tering data are plotted as qR_θ/Kc against $q\langle S^2\rangle^{1/2}$ (where $\langle S^2\rangle^{1/2}$ is the apparent radius of gyration at the concentration c in Figure 3).

In this graph, known as Holtzer plot,[13,14] both experimental and extrapolated curves reach, at large q, a constant plateau of the same value. From the theory of rigid rods the magnitude of this plateau gives the linear mass density M_L

$$qR_\theta/\pi Kc \rightarrow M_L$$

which represents the mass per unit length of the rod. This value can be calculated, in an approximate fashion, for a single-stranded chain of N-S-1 by using the repeating unit molecular weight M_0 = 1542 and the corresponding length of 1.92 nm (ref 15, p 84) (the repeat length of succinoglycan helices) which gives M_L(single strand) = 803 nm⁻¹. The ratio M_L(exptl)/M_L(single) permits the estimation of the number of laterally interacting strands of the ordered N-S-1 conformation in solution, which is found to be n-(strands) = 1.3.

The Holtzer plot exhibits a maximum at low q values as predicted by the Koyama theory for semiflexible chains.[16] As shown by Schmidt et al.[14] and as recently discussed for other microbial polysaccharides,[17,18] the ratio of the height of the maximum to the asymptote is a function of the number of Kuhn segments N_K per chain, whose length is a characteristic parameter of chain stiffness. This maximum occurs at $q\langle S^2\rangle_z^{1/2}$ = 1.73 for N-S-1, as expected for a polydisperse sample with M_w/M_n = 2. The obtained Kuhn length can be cross-checked by calculating the radius of gyration, assuming the most probable Schulz–Flory distribution, and using the formula by Benoit and Doty for wormlike chains:[19] an excellent agreement results from a comparison between the experimental radius of gyration and the calculated one (see Table I).

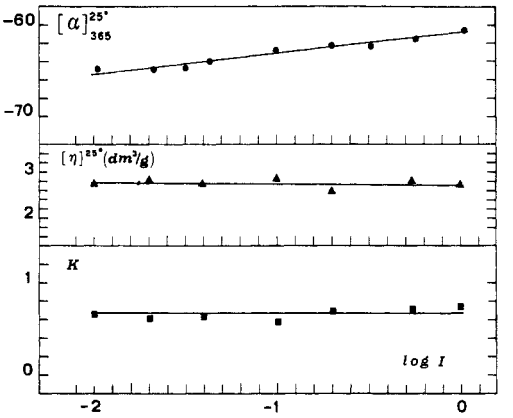

Figure 4. Dependence of N-S-1 optical activity (λ = 365 nm; polymer concentration 1.3 mequiv/dm³), intrinsic viscosity, and Huggin's constant, k, on the ionic strength, I(NaCl), at 25 °C.

A further check has been made for the angular dependence of the scattered light by using the equation of Koyama for polydisperse semiflexible chains[16] (see the full line in Figure 3).

All properties of N-S-1 which have been derived from the Zimm and Holtzer plots are collected in Table I, in comparison with the light scattering results of xanthan,[17] another very stiff ionic polysaccharide. It is worth noting the high value of l_K for both polysaccharides.

(b) Viscosity and Optical Activity (25 °C). As shown in Figure 4, the optical activity of N-S-1 at 25 °C and λ = 365 nm is only slightly influenced by changing the ionic strength, I(NaCl): in addition, the intrinsic viscosity and Huggin's constant characteristic of our N-S-1 sample are almost unaffected on going from 0.01 M up to 1.0 M NaCl (always at 25 °C).

These results strongly suggest that the solution state of the N-S-1 chains as derived from light scattering data (0.1 M NaCl, 25 °C) is basically unperturbed by changes in ionic strength at room temperature.

In view of the regular primary structure of N-S-1, the information above suggests that the polysaccharidic chains may be in an ordered conformational state in aqueous NaCl and 25 °C: from a possible extrapolation of the viscosity and optical activity data of Figure 4, this might hold true also in water.

2. The Influence of Heating ↔ Cooling Cycles on the Solution Properties of N-S-1. The study of changes in dilute solution properties of N-S-1 during the course of as well as following thermal cycles has lead to a number of interesting observations. The latter are illustrated below according to the experimental technique employed.

In all cases, the thermal cycles were performed as follows: (1) heating from room temperature to ca. 85 °C in about 5 h; (2) cooling back to 25 °C at approximately the same rate; (3) annealing at 25 °C (from 12 h to many days).

(a) Viscosity. As shown by the data reported in Figure 5, the viscosity of our succinoglycan sample is reduced as a result of a single thermal cycle: in practice, the intrinsic viscosity (0.01 M NaCl and 25 °C) is approximately halved. This is in qualitative agreement with that reported by others[15] using a different succinoglycan sample.

However, successive thermal cycles, always carried out as specified above, do not lead to further reduction in viscosity: as a matter of fact, the final intrinsic viscosity

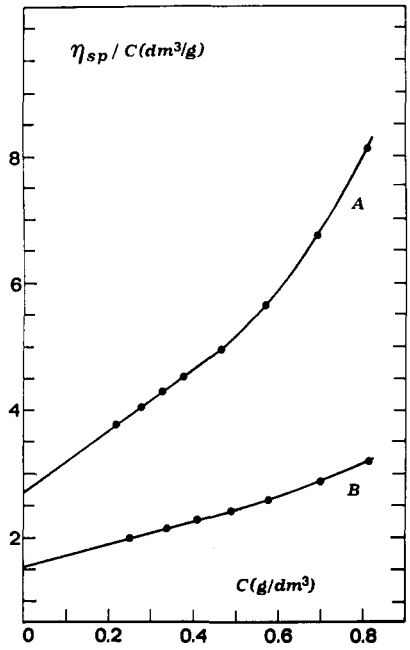

Figure 5. Reduced specific viscosity of succinoglycan at 25 °C in 0.01 M NaCl as a function of polysaccharide concentration (g/dm³): (A) N-S-1 (sample never heated above 25 °C); (B) sample after a thermal cycle (see section 2) and annealing overnight at 25 °C.

([η] = 1.5 dm³/g; see Figure 5) remains practically *constant* and independent of the "annealing" time (even weeks) at 25 °C. The same results have been obtained working at different ionic strengths, up to 2 M NaCl, at which practically the same final [η] value was reproduced.

These data clearly suggest that N-S-1 chains would undergo an irreversible reduction in apparent molecular weight only after a first, single thermal cycle in aqueous NaCl. This phenomenon should not be ascribed to random chain scission promoted by free radical attack, in view of the insensitivity of the intrinsic viscosity to additional thermal treatments and because it can be exactly reproduced working in the absence of oxygen and in the presence of a radical scavenger (*p*-methoxyphenol).

NMR and potentiometric titration data also show that the thermal treatments specified above do *not* change the pyruvyl and succinate group content of our sample, at least within the experimental error of the two techniques.

An additional, empirical observation is that while succinoglycan kept in solution after a thermal cycle does not recover its initial viscosity, once it is dialyzed and freeze-dried it then dissolves with difficulty yielding a slightly cloudy solution even more viscous than the original one (that of "never heated" succinoglycan, i.e., N-S-1) for the same polymer concentration. A new thermal cycle on the latter solution reestablishes conditions outlined above, that is: [η] drops back to the final, stable value of ca. 1.5 dm³/g at 25 °C. On the other hand, if a succinoglycan–NaCl solution is freeze-dried without prior desalting, the polymer readily dissolves in aqueous NaCl and directly exhibits the "final" intrinsic viscosity repeatedly specified above.

This set of observations is, in our opinion, quite relevant in an attempt to interpret the results presented in what follows.

(b) Optical Activity and Circular Dichroism. Typical optical activity–temperature plots obtained by using N-S-1 (0.1% w/v) in water and in different NaCl concentrations are reported in Figure 6. The experiments were carried out following the heating–cooling protocol already indicated at the outset of section 2.

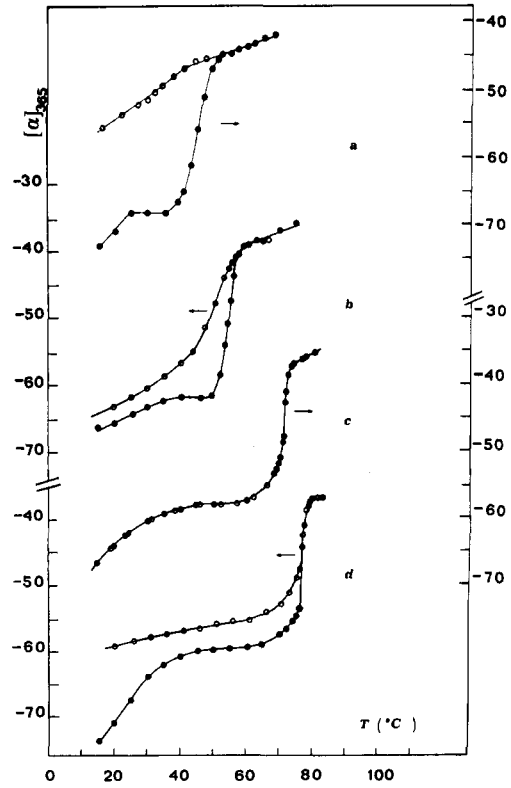

Figure 6. Dependence of succinoglycan optical activity on temperature at different ionic strengths I(NaCl): (a) 0.0; (b) 0.01; (c) 0.55; (d) 2.23. Polymer concentration, 1.3 mequiv/dm³; (●) heating; (○) cooling.

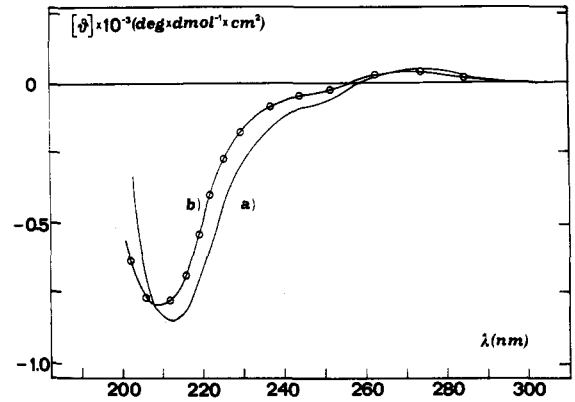

Figure 7. Circular dichroism spectra of succinoglycan at 25 °C in (a) water and (b) 0.1 M NaCl (open circles after thermal cycle and annealing at 25 °C for ca. 12 h). Polymer concentration, 1.3 mequiv/dm³.

Plots of Figure 6 exhibit three main features: (1) their trend is that normally associated with highly cooperative, thermally induced conformational transitions of polysaccharide chains in aqueous solution; (2) the temperature at the inflection points (T_M) of the sigmoidal [α] versus T curves (heating) increases with increasing ionic strength; (3) the cooling curves may present hysteresis, the phenomenon being particularly evident in water, absent in 0.55 M NaCl, and again clearly discernible in 2.23 M NaCl. Inspection of a number of additional data, not reported here for brevity, reveals that hysteresis is not observed for NaCl concentrations in the range $0.1 < M < 1.0$. After a thermal cycle, leaving the sample at 25 °C long enough (maximum of ca. 5 days), the initial optical activity is *nearly exactly recovered* in all cases.

Analogous conclusions can be reached by considering the circular dichroism (CD) results illustrated in Figure 7,

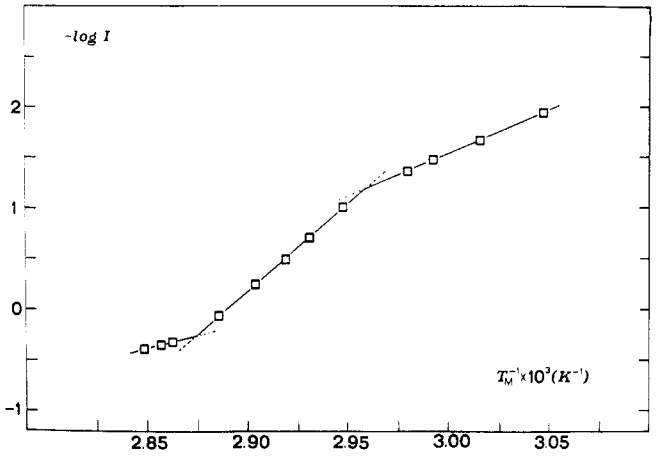

Figure 8. Relation between T_M ("melting" temperature) of succinoglycan (determined at the inflection points of the optical activity against T plots; see, for example, Figure 6) and the ionic strength I(NaCl).

according to which the same spectrum is obtained for succinoglycan in 0.1 M NaCl before *and* after a thermal cycle. Moreover, the CD spectrum of N-S-1 results are only scarcely influenced by ionic strength.

In conclusion, it appears that the thermally induced conformational change of our succinoglycan sample in aqueous NaCl is completely reversible as far as the chiroptical features of the polysaccharidic chains are concerned.

It seems logical to assume, also in view of the discussion in the preceding section (2-a), that such a conformational change is a typical order → disorder process. We propose that the initial solution state of N-S-1 freshly dissolved in aqueous NaCl corresponds to partially aggregated, single-helical chains whose conformation (and degree of intermolecular aggregation) is scarcely dependent on NaCl concentration (25 °C).

Upon heating of the solution above ca. 85 °C, a state in which succinoglycan chains would be essentially singly dispersed and lack long-range conformational order largely prevails. Due to the screening exerted by added Na^+ counterions on N-S-1 fixed charges (see also section 2-c), the conformational order → disorder change takes place at temperatures (T_M) increasing with ionic strength I, as is commonly observed with other biopolyelectrolytes.

In the case of succinoglycan, this finds representation in the $1/T_M$ against log (I) plot of Figure 8. The latter plot, contrary to what is normally found, exhibits three distinct linear regions, the middle one corresponding to the NaCl concentration range where no hysteresis is observed in the [α] versus T cooling curves (the data of Figure 8 are further discussed in section 2-c). Considering all information presented above, we propose that on cooling a succinoglycan aqueous solution from ca. 85 °C back to room temperature the polysaccharide backbone helical conformation is reestablished (optical activity and circular dichroism data), more or less slowly depending on NaCl concentration (the process might just be faster for NaCl concentrations in the 0.1–1.0 M range), but the degree of chain aggregation is finally markedly and irreversibly reduced (viscosity data). It should also be pointed out that on submitting a given succinoglycan solution in NaCl to a second thermal cycle, the associated T_M value is reproduced, but the hysteresis, if any, is largely diminished or completely abolished.

(c) Differential Scanning Calorimetry. The results of differential scanning calorimetry (DSC) experiments carried out by using aqueous 0.2% w/v succinoglycan in

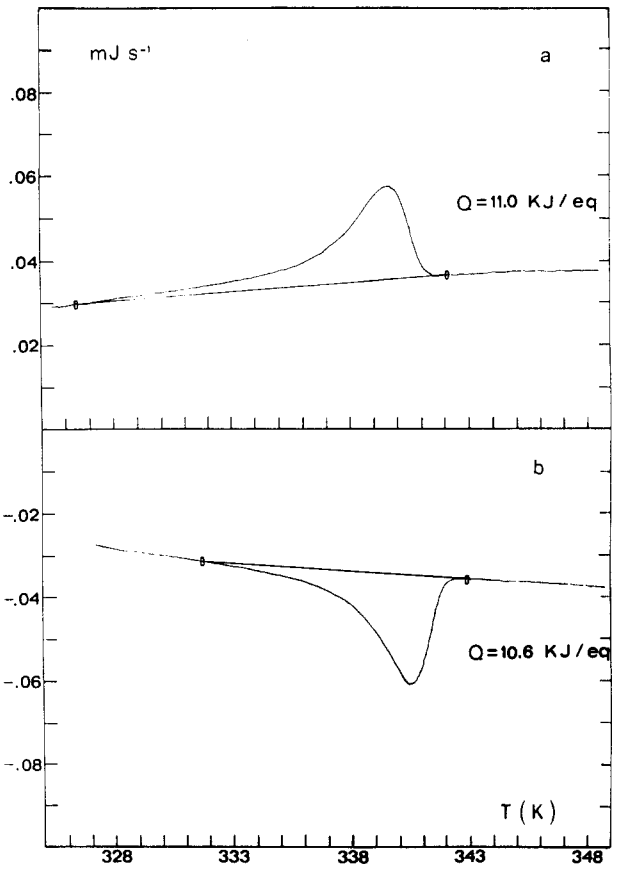

Figure 9. Differential scanning calorimetry curves for aqueous succinoglycan (0.2% w/v in 0.2 M NaCl): (a) heating; (b) cooling. Each thermogram is the average of 14 thermal cycles. Scan rate, 0.2 °C/min.

0.2 M NaCl are reported in Figure 9.

Three particularly important features stem from these experiments: (1) The peak temperatures on heating and on cooling are almost coincident (67.5 and 66.7 °C, respectively); this is in very good agreement with what is found in the [α] versus T experiments for the same NaCl concentration (T_M = 68 °C, no hysteresis). (2) The area under the two DSC plots of Figure 9 is practically the same and corresponds to a heat exchange of 11.0 kJ/equiv of polymer. (3) Repeated DSC runs on the same solution reproduce the results indicated in (1) and (2) and yield an average value for the enthalpy ($\Delta_M H$) of the succinoglycan order → disorder transition of 10.6 ± 0.4 kJ/equiv of polysaccharide.

Additional DSC experiments performed by using different NaCl concentrations basically confirm observations (1)–(3) with peak temperatures closely corresponding to the T_M figures derived from the optical activity experiments.

Finally, it is interesting to compare predictions based on Manning's polyelectrolyte theory[20–22] with our experimental evidence discussed above and in section 2-b. According to this theory, the value of $\Delta_M H$ (in the units indicated above) associated with the "melting" of the ordered conformation of a charged biopolymer in dilute solution can be correlated with the change in linear charge density experienced by the chains and with the slope of the corresponding I/T_M against log (I) linear plot on the basis of the equation

$$\Delta_M H/(\mathrm{d}\log(I)/\mathrm{d}(1/T_M)) = -9.57 f(\xi_C,\xi_H) \qquad (1)$$

in which ξ_C and ξ_H are the linear charge density parameters characteristic of the disordered (C) and of the ordered (H)

chain states, respectively: the form of the function $f(\xi)$ depends on the values of both parameters.

In dilute aqueous solution at 25 °C, it is, by definition, $\xi = 0.714b$, where b (nm) is the average, projected distance between neighboring fixed charges along the chains. In our case, with 1.92 nm as the repeat length of succinoglycan helices (ref 15, p 84), which corresponds to $b = 0.96$ nm (single helices), and the assumption $b = (2.06/2)$ nm for the disordered, fully extended state, the two limiting ξ values would be 0.74 and 0.69, respectively.

The $f(\xi)$ function then simply reduces[22] to $(\xi_C - \xi_H) = -0.05$, and the right-hand side of eq 1 is equal to 0.48.

According to data reported in Figure 8, confining attention to the middle linear portion of the plot (absence of hysteresis; see section 2-b) and introducing our calorimetric $\Delta_M H$ value together with the slope of the said portion, the left-hand side of eq 1 is equal to 0.57.

In view of the uncertainties, traceable to the experimental errors, the approximate nature of the theory underlying eq 1, and the approximate figures chosen for the limiting b values, the agreement may be considered fair and, thus, in support of the single-helix hypothesis for N-S-1 (and for "annealed" succinoglycan) in aqueous NaCl.

It has to be remarked that using a different succinoglycan sample, other authors[15] have determined for the enthalpy of melting (in ca. 0.2 M NaCl) a value which is seven times higher than ours. We have no explanation for such a discrepancy with our results.

Concluding Remarks

A possible rationalization of the set of experimental evidence discussed in this paper may be achieved, in our opinion, on the basis of the following hypotheses.

N-S-1 chains in aqueous media and at room temperature are in a helical conformation and give rise to aggregation via lateral, partial association mediated by the side chains. In order to interpret the observed mass-per-unit-length ratio of Table I, one may assume a limiting model in which approximately one-third in length of each rigid, helical N-S-1 chain would be engaged, on the average, in pairwise intermolecular interactions. This situation would prevail at room temperature in water and in aqueous NaCl.

On heating, chain association is markedly weakened and, eventually, a conformational transition takes place, leading to single, expanded chains.

Subsequent cooling (aqueous NaCl) may reestablish, more or less readily depending on salt concentration, the helical conformation of the succinoglycan backbone: however, the presence of a simple electrolyte would not allow lateral chain association, probably by inducing the side chains to assume a state more compact than that prevailing before thermal treatment. Desalting the polysaccharide solutions permits expansion of the lateral chains with a renewal of partial intermolecular association.

These hypotheses can qualitatively explain the experimental facts illustrated in sections 1 and 2 with one additional assumption; namely, the possible perturbation in chiroptical properties associated with the alleged change in conformation experienced by a fraction of the succinoglycan side chains during the thermal cycles is negligible in comparison to the major perturbation consequent to the backbone conformational transition.

Indeed, the picture submitted above is at the molecular level a gross simplification of an obviously more complex reality in which other factors, such as water "structure" and electrolyte–water–hydrophylic polymer interactions,[23] certainly play an important role.

In addition, an experimentally based explanation remains to be found for the peculiar change in the extent of the hysteresis which characterizes succinoglycan thermal transition on going from dilute to concentrated NaCl solutions (section 2-b) and which finds a counterpart in the segmented plot of Figure 9 (sections 2-b and 2-c).

A systematic kinetic investigation should help in understanding this important aspect.

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