Carbohydrate Research, 231 (1992) 117–135
Elsevier Science Publishers B.V., Amsterdam

Solution conformation and properties of the galactoglucan from *Rhizobium meliloti* strain YE-2(S1)

Attilio Cesàro [a], Gianpaolo Tomasi [a], Amelia Gamini [a], Sandro Vidotto [a] and Luciano Navarini [b,1]

[a] *Department of Biochemistry, Biophysics, and Macromolecular Chemistry, University of Trieste, 34127 Trieste (Italy)*
Laboratory of Technological Biopolymers, POLYbiòs Research Centre, Area di Ricerca, Padriciano 99, 34012 Trieste (Italy)
[b] *International Centre for Pure and Applied Chemistry, Area di Ricerca, Padriciano 99, 34012 Trieste (Italy)*

(Received October 7th, 1991; accepted January 3rd, 1992)

ABSTRACT

The physicochemical solution properties of the galactoglucan excreted by *Rhizobium meliloti* strain YE-2(S1) have been investigated by capillary viscometry, potentiometric titration, isothermal mixing microcalorimetry, and circular dichroism. Potentiometric and chiro-optical data, as a function of the degree of ionisation, indicate the absence of a co-operative conformational transition. Solution properties, as a function of ionic strength and temperature, suggest that the galactoglucan adopts a disordered conformation characterised by moderate flexibility. Polyelectrolyte theory is used to fit the enthalpy of dilution data with a suitable linear charge-density parameter. Conformational calculations and chain modelling, using molecular mechanics, give an unperturbed characteristic ratio, (C_∞) of 20, which was smaller than that estimated from intrinsic-viscosity and molecular-weight data for an expanded-coil chain model.

INTRODUCTION

Although it is asserted that biological and technological properties are closely related to the conformational structure of a biopolymer in its functional state, the number of physicochemical studies of new microbial polysaccharides is rather

[1] Present address: Laboratory of Technological Biopolymers, POLYbiòs Research Centre, Area di Ricerca, Padriciano 99, 34012 Trieste, Italy.
Correspondence to: Professor A. Cesàro, Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecule, Universita degli Studi di Trieste, Via Valerno 22, I-34127 Trieste, Italy.

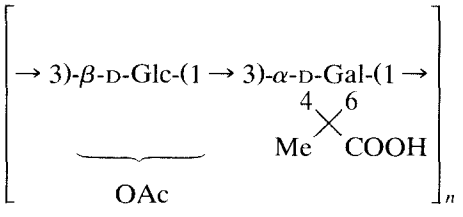
limited and attention has been focused on commercially viable polymers, e.g., xanthan and the gellan-like group[1].

Micro-organisms of the genus *Rhizobium* are of great interest, not only for their important role as nitrogen-fixing bacteria, but also for their production of oligo- and poly-saccharides[2]. The exopolysaccharides are believed to play a role in determining the specificity of symbiosis with the host plant. Bacteria induce the formation of nodules and their exopolysaccharides appear to bind to lectins secreted by plants. Many of the *Rhizobium* polysaccharides give highly viscous aqueous solutions with rheological properties suitable for technological applications. This behaviour is attributed to polysaccharides termed "succinoglycans". Rhizobia belonging to the *Agrobacterium–R. meliloti* group usually produce a high molecular weight succinoglycan[3].

Mutants of native strains have different patterns of polysaccharide production[4]. Among these strains, *R. meliloti* mutant YE-2(S1) excretes a mixture of soluble polysaccharides[5], including a succinoglycan and a galactoglucan, with a glucose : galactose ratio of 1 : 1 and having acetyl and pyruvic substituents (**1**). Low molecular weight units of the succinoglycan, but not of the galactoglucan, are excreted. It has been concluded[5] that the biosynthesis pathways for the succinoglycan and galactoglycan are the same. Low osmolarity of the medium increases the proportion of the galactoglucan, whereas high osmolarity (> 0.4 M) promotes formation of the succinoglycan[6]. A pure single polysaccharide has not been obtained and the whole production depends on the age of the culture. An investigation of the viscoelastic properties of the mixture of exocellular polysaccharides revealed behaviour typical of an entanglement system for the galactoglucan-rich fraction[7]. The viscoelastic properties change abruptly when there is a particular proportion of the succinoglycan, producing results typical of a "weak gel" system[7].

Apart from possible commercial applications of the two polysaccharides, it is important to determine the mechanism by which the bacteria switch the production of the exocellular polysaccharides.

It is possible that differences in the physicochemical behaviour of the two polysaccharides could be responsible for the changes in the proportion excreted by the micro-organisms in response to changes in the conditions of growth. In this context, determination of the physicochemical properties for the two polysaccharides is important and we now report on such properties of the galactoglucan.



1

THEORETICAL APPROACHES

Polyelectrolytic theory.—In solution, the charges on a polyelectrolyte are neutralised typically by small ions (counterions). An accurate description of the stoichiometry and mode of binding of the counterions to the polyion has been a goal of experimental and theoretical studies[8–13].

In order to relate such thermodynamic parameters as enthalpy data (dilution, mixing, dissociation, etc.) to the charge density on the polymer, the basic approach of the counterion-condensation theory based on the simplest chain model characterised by the linear extended conformation has been used[12,13]. This model assumes that the intense electric field generated by the charged groups on a linear polyion has roughly cylindrical symmetry, coaxial with the contour of the polymer chain. The ionic interactions extend over only ~ 3 nm for an ionic strength of 0.001 M. This assumption can be relaxed and the flexibility of the chain has been included in more recent calculations[14].

According to the assumptions of the original model[8–13], the charged groups are represented as point charges with uniform spacing (b). As a consequence of the binding of counterions (termed condensation for the fraction intimately bound to the polyelectrolyte), each univalent charge point bears an effective charge of $|(1-r)q|$, where q is the electron charge and r is the fractional extent of counterion binding. In the following, the polyelectrolyte is a polyanion and both the fixed charges and the counterions are monovalent.

The ionic contribution to the free energy associated with each polyion has been computed and extended to other thermodynamic functions[13].

The (excess) electrostatic enthalpy of the polyelectrolyte solution as a function of the dimensionless linear charge-density parameter ξ and of the polyion concentration C, which enter into the Debye screening parameter (defined below), has been derived[12] as follows:

$$H^{el} = \left(\frac{\partial G^{el}/T}{\partial 1/T}\right)_{n,b} = -\tfrac{1}{2} n_e RT\xi \left(1 + \frac{\partial \ln D}{\partial \ln T}\right)\left[2\ln(1-e^{-Kb}) + \frac{Kb}{e^{Kb}-1}\right], \quad (1)$$

where $\xi = l_B/b$, $l_B = e^2/DkT$ is the Bjerrum length (Å), e is the elementary charge, D is the bulk dielectric constant of the medium, T is the absolute temperature, k is the Boltzmann constant, and b (previously defined) is expressed in Å.

Therefore, the expression for the (electrostatic) enthalpy change upon mixing n_e moles of polymeric salt with pure solvent, in order to change the polymer concentration from C_i to C_f, is, for $\xi < 1$,

$$\Delta_{dil}H = H^f - H^i = -1/2RT\xi^m\left(1 + \frac{\partial \ln D}{\partial \ln T}\right) \cdot \left\{2\ln\frac{1-e^{-K_f b}}{1-e^{-K_i b}} + \frac{K_f b}{e^{K_f b}-1} + \frac{K_i b}{e^{K_i b}-1}\right\} \quad (2)$$

where K^{-1} is the Debye length, defined as $K^2 = 4\pi l_B N I_{i,f}/10^{27}$, $I_{i,f}$ is the total initial (i) or final (f) molar concentration of ions in the medium, and m = 1. For salt-free solutions, $I_{i,f} = C_{i,f}$.

In the limit of very dilute solutions and, more generally, when $Kb \ll 1$, then the terms within the brackets reduce eq *2* to

$$\Delta_{dil} H = -\tfrac{1}{2} RT \left(1 + \frac{\partial \ln D}{\partial \ln T}\right) \xi^m \ln \frac{C_f}{C_i} \qquad (3)$$

where, as before, m = 1 for $\xi < 1$.

The simplified eq. *3* shows why the enthalpy data of dilution are usually reported as a function of the log of the polyelectrolyte concentration, *C*. Furthermore, it shows that the normalised value of the slope (i.e., the constant part of equation *3*) is equal to 1058 J/mol (for univalent charges at 25°).

Conformational analysis.—Conformational analysis, based on approximate (unrefined) potential functions, is useful[15] for studying the relationships between the structure and such macromolecular properties of polysaccharides as flexibility and extension of the chain. Several such studies of homopolysaccharides have appeared in the literature, including the effect of solvent on the chain conformation[16].

In the present study, the initial problem was the selection of the (internal) atomic coordinates for the two sugar residues. The geometry of the β-D-glucopyranose moiety was taken from that proposed[17] and that of the α-D-galactopyranose moiety was obtained by epimerisation[18] of the α-D-glucopyranose residue. In addition, both the co-ordinates and the charges of the atoms for the disaccharide repeating unit, with the pyruvic acid 4,6-linked to the α-D-galactopyranosyl residue, were obtained from the minimisation of the 3-*O*-[4,6-*O*-(1-carboxyethylidene)-α-D-galactopyranosyl]-β-D-glucopyranose structure, using the packages MOPAC and DISCOVERY. The configuration of the pyruvic acetal moiety is assumed to be *R*, since ab initio calculations indicate this configuration to be thermodynamically the most stable; the *R* configuration has been observed for the pyruvic group 4,6-linked to D-galactopyranosyl residues[19].

In order to calculate the conformational energy of the disaccharide unit, the pyranose rings were assumed to be rigid while the glycosidic torsional angles (Φ, ψ) were varied in the range −180 to +180° in increments of 10°. The potential functions included van der Waals and coulombic contributions. The van der Waals contributions were evaluated as described[15] and the coulombic contributions were calculated from the partial atomic charges obtained from standard bond dipoles and bond lengths. For the residues of the polysaccharide with pyruvic acetal residues, the coulombic contributions were obtained directly from the minimisation.

Evaluation of the conformational energy of the unit of the chain is the basic step for computing all macromolecular properties of a chain with dp *n*. The most significant characteristics are given by the unperturbed dimensions of a chain, expressed by the mean-square end-to-end distance ($\langle r_n^2 \rangle_0$) where the unperturbed

state is signified by the subscript zero. However, it is more common to use the adimensional, asymptotic value of the characteristic ratio (C_n) defined as:

$$C_\infty = \lim_{n\to\infty} C_n = \lim_{n\to\infty} \langle r_n^2 \rangle_0 / nL^2. \quad (4)$$

This quantity reaches an asymptotic value, since it is normalised for the virtual bond length L and by the number of residues n, and can be calculated by standard matrix methods[20]. The same procedure can also be used to obtain the chain-length dependence of C_n.

In addition, Monte Carlo techniques[21] are often employed, not only to compute $\langle r_n^2 \rangle_0^{1/2}$ and the characteristic ratio, but also to obtain "snapshots" of the chain and normalised distribution functions of the end-to-end distance for different chain lengths.

Another quantity of interest for the study of flexibility and directionality of the chain is the correlation function (f_n), defined as the mean projection of a unit vector along the nth virtual bond of the chain onto that aligned with the initial virtual bond. The periodic pattern of the correlation function (e.g., for the amylose chain) is a consequence of the pseudohelical trajectory of the chain. The decay rate of this function, which can be defined as the number of residues (x_c) necessary in order to have $f_n < e^{-1}$ for $n > x_c$, is a measure of the directional persistence in residue units.

A description for calculating these chain-dimensional properties, including a discussion on the potential function and its parametrisation and on the averaging procedures, has been reported[21]. Most of the software package, which has also been used to include the solvent effect[16], was originally designed for a homoglycan and had to be rewritten in order to increase its capability and to extend its application to the galactoglucan, but the architecture was retained.

EXPERIMENTAL

Materials.—R. meliloti, slimy variant strain YE-2(S1), was grown under the conditions described[6] at the Department of Microbiology, Agricultural University, Wageningen (The Netherlands). The galactoglucan was recovered after 5 days of growth in a medium without added salt. A solution of the crude polysaccharide in bidistilled water (*c* 2 g/L) was dialysed against 2.5 mM citric acid in M NaCl, then exhaustively against bidistilled water, filtered (5.0 μm), and freeze-dried. The product contained 15% of water (Karl-Fisher titration).

Solutions prepared by stirring the polysaccharide in bidistilled water overnight at room temperature were mixed with appropriate solutions of NaCl or $NaClO_4$ to achieve the desired concentration of polysaccharide and ionic strength.

Aqueous solutions of the polysaccharide in the acid form were obtained by dialysis in sequence against 0.1 M acetic acid (twice), 0.01 M HCl, then bidistilled water exhaustively. The concentration of the acidic polysaccharide was determined by potentiometric titration. Solutions with different degrees of ionisation (α) were

obtained by the progressive addition of 0.1 M NaOH to the aqueous solution of the acid form of the polysaccharide.

Two different samples (A and B), recovered after the purification procedure, were identical except for molecular weight.

The molecular weight and the molecular weight distribution of samples A and B were established by gel-permeation chromatography on columns of B-800P, OM-pak B-806, and B-803 SHODEX in series, using a low-angle laser-light-scattering (LALLS) detector (Chromatix CMX100) and a refractometer (Waters 410) in line. Each 1.1% polysaccharide solution was filtered through a 0.45-μm filter (Millipore), then injected into the chromatograph and eluted with phosphate buffer (pH 7). The results indicated a $\langle M_w \rangle$ of 7.77×10^5 and 2.67×10^5 and polydispersities of 2.22 and of 3.95 for samples A and B, respectively. Sample B was assumed to be a partially depolymerised galactoglucan because of its higher polydispersity. Wherever possible, the measurements were carried out on sample A.

Capillary viscometry.—A Shott–Geräte automatic viscometer with an Ubbelohde capillary (0.46 mm) was used. A 0.17 g/L solution was prepared by vigorously stirring the freeze-dried polysaccharide in bidistilled water for 24 h. Solutions with different ionic strengths were prepared from this stock solution by 1:1 dilution with aq $NaClO_4$ at double the final required concentration. Each solution was dialysed against the aq $NaClO_4$ used as solvent in the determination of the intrinsic viscosity, performed at 25°. Each solution to be injected into the viscometer was filtered through a 0.45-μm Millipore filter. At least seven flow times were programmed for each dilution, of which five were used with an agreement to within ± 0.1 s. All flow times and relative concentrations were processed to include kinetic energy corrections with a computer package VISCO on a VAX station, and taking the double extrapolation of the Huggins' and the Kraemer plots.

Potentiometric titrations.—The protonated form of the polysaccharide was prepared by dialysis against aqueous acid solutions and then bidistilled water, as described above. Solutions with different concentrations of polysaccharide and acid were used, since these proved to affect the extent of chain aggregation. The pH titrations were carried out with a Radiometer PHM-52 digital pH meter with a glass–calomel GK 2301C combined electrode.

Isothermal mixing microcalorimetry.—Experiments were performed at 25° using an LKB 10700-2 batch-type isothermal microcalorimeter, equipped with gold cells. In each experiment, the solution of the polysaccharide (2 mL, 9.93 g/L) prepared in the usual way was mixed with water (2 mL). The twin-cell system was balanced with a reference mixing of equal volumes of pure solvent. The calorimeter signal, amplified with a Keithley 150B microammeter, was recorded and the corrected total heat change was evaluated and related to the dilution of the polysaccharide from the initial (C) to the final concentration ($C/2$). The resulting solution was used for successive dilution experiments, until a detectable heat was measured within the limits of instrument sensitivity. The final results were plotted as $\Delta_{dil}H$ vs. log C.

Circular dichroism.—A Jasco Model J-500A dichrograph was used equipped with a Jasco DP-500N data processor. The molar ellipticity ($[\theta]$) was calculated on the basis of the polysaccharide concentration (C) expressed in equiv/L (or, where specified, in g/L).

RESULTS AND DISCUSSION

Characterisation of the polysaccharide.—Cultures of the *R. meliloti* mutant YE-2(S1) produced a mixture of two exopolysaccharides, identified as EPS-I (succinoglycan) and EPS-II (galactoglucan). A medium of low ionic strength favoured a high content of galactoglucan. As reported[6], octasaccharide repeating units and higher oligosaccharides of EPS-I and EPS-II were the only components of the secreted carbohydrates.

Simple precipitation with ethanol effected only a rough separation between the high- and low-mass components. In order to minimise the contamination by EPS-I, EPS-II, isolated from a medium of low ionic strength at an early stage of growth, was used. The polysaccharide used throughout the work was isolated from the mixture (sample 00-5d).

Chemical analysis revealed up to 25% of EPS-I in a 10-day culture (sample 00-10d) and ~ 20% in a 5-day culture (sample 00-5d). After dialysis of the mixture of polysaccharides against 2.5 mM citric acid, M NaCl, and water (several times), the amount of "contaminant" was reduced to < 10% as determined by gel-permeation chromatography (see Experimental). In fact, a small broad peak was present in the low molecular weight range and assumed to be the non-dialysable fraction of EPS-I; it gave no detectable scattering. On the assumption that the refractive index is proportional to the concentration of the solute, the total amount of this contaminant, with molecular weight of $\sim 2 \times 10^4$ was estimated to be 9%. The influence of this proportion of low molecular weight EPS-I is negligible for most of the "relative" measurements, but it had to be taken into account as a possible source of error in the calculation of absolute quantities.

As an example of this aspect, the CD spectra were recorded for samples of exocellular polysaccharides, containing various proportions of EPS-I (succinoglycan) and EPS-II, and for the sample used in this work (Fig. 1). Spectra of mixtures (samples 06-7d and 04-7d) containing a high proportion of EPS-I are characterised by a distinct (negative) band centered at 210 nm. The crude sample 00-7d still shows a shoulder between 200–210 nm, whereas the spectrum of the purified sample 00-5d has a well-defined band at ~ 225 nm and reaches positive values of $[\theta]$ below 205 nm. Different conclusions may be reached if the CD signal is monitored at a different wavelength as a function of an independent variable, such as temperature, ionic strength, or pH.

Not only are the chemical structures of the two polysaccharides markedly different, but also the number of the acidic groups in the repeating unit, which gives an equivalent weight of 754 and 436 for the succinoglycan and the galactoglu-

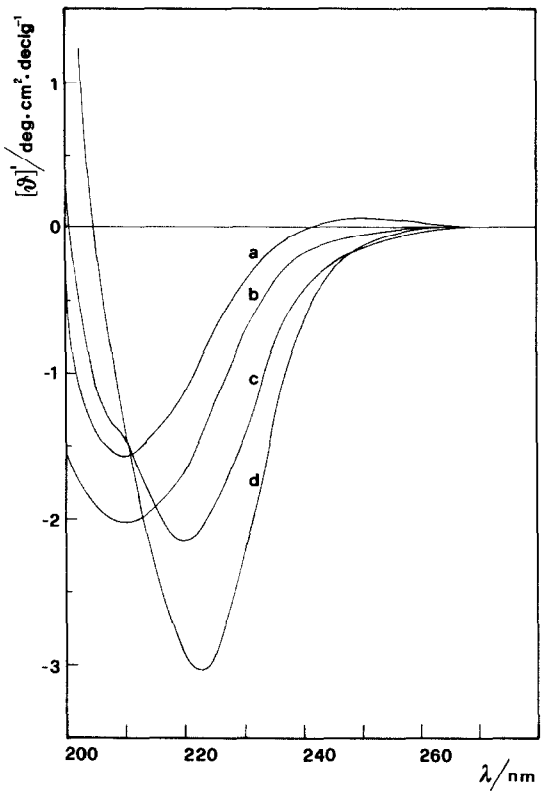


Fig. 1. CD spectra of the galactoglucan and succinoglycan mixture isolated from culture broths of different ionic strengths (0.6, 0.4, and 0.0 M NaCl) and age (7 and 5 days); samples: 06-7d (a), 04-7d (b), 00-7d (c), 00-5d (d).

can, respectively. When normalised as in Fig. 1 for the total concentration of solute (g/L), the spectra change in a regular fashion and, given the fractions of the two polysaccharide components, the CD spectra of the pure galactoglucan and that of the pure succinoglucan may be extrapolated.

Dilute solution properties.—(a) Potentiometric titrations and pH-dependence of circular dichroism. The presence of the pyruvic group in the repeating unit gives the galactoglucan its polyelectrolyte properties. Moreover, the pyruvic and acetate groups together constitute the chromophore, and the CD spectrum shows a negative band with the maximum at ~ 225 nm. Therefore, the pH-dependence of the proton dissociation constant and of the CD bands were studied in order to assess the occurrence of abrupt changes of conformation as a function of the density of electrostatic charge on the polysaccharide chain.

Fig. 2 shows the potentiometric titration curves for two different concentrations of polysaccharide (1.11×10^{-3} and 0.39×10^{-3} equiv/L) and ionic strengths (water and 0.1 M NaCl). There is a negative slope in the initial portion of the pK_a

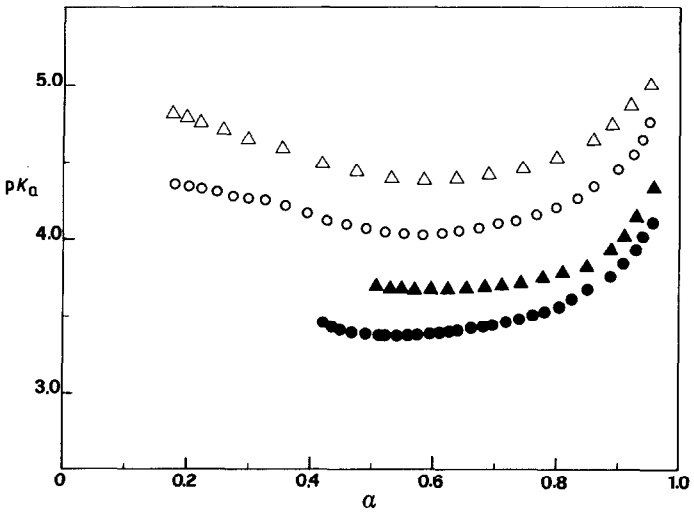


Fig. 2. Dependence of the apparent pK_a on α for solutions of the acid form of the galactoglucan at 25°: open symbols in water, full symbols in 0.1 M $NaClO_4$, C 1.1×10^{-3} (○, ●) and 0.39×10^{-3} (△, ▲) equiv/L.

curves (at low α values) obtained for solutions in water. This behaviour can be explained, also theoretically[22], by a disaggregation process which occurs on ionisation of the carboxyl groups, leading to a decrease of the net-charge density. The experimental values were reproducible even when slightly opalescent solutions at higher concentrations of polysaccharide were used. The extent of chain aggregation was reduced when low concentrations of polysaccharide were used. The addition of salt, as well as an increase in the concentration of the polysaccharide, makes the solution more acidic, as shown by the decrease in the pK_a values. The pK_a curves shown in Fig. 2 cannot be defined as "regular", as with simple polycarboxylic chains. However, the presence of the reported aggregative process at low pH completely masks possible (if any) conformational changes which could modify the value and distribution of the charge density.

The CD spectra were recorded for solutions in water as a function of the degree of ionisation (α). Fig. 3 shows the change of the molar ellipticity ($[\theta]_{225}$) at 225 nm as a function of α. The dependence is almost linear, indicating that, at any α value, $[\theta]_{225}$ is simply the sum of the contributions from the protonated and the unprotonated carboxyl chromophores. Although a slight downward curvature can be detected in the trend shown in Fig. 3, there is no evidence of spectral perturbations due to conformational change. The hypothesis of a simple equilibrium between two different chromophores is confirmed by the presence of an iso-dichroic point centered at 238 nm.

From this set of data as a function of the degree of ionisation (α), it is concluded that the average conformation of the exopolysaccharide, although necessarily a function of the pH, does not undergo a co-operative transition. No

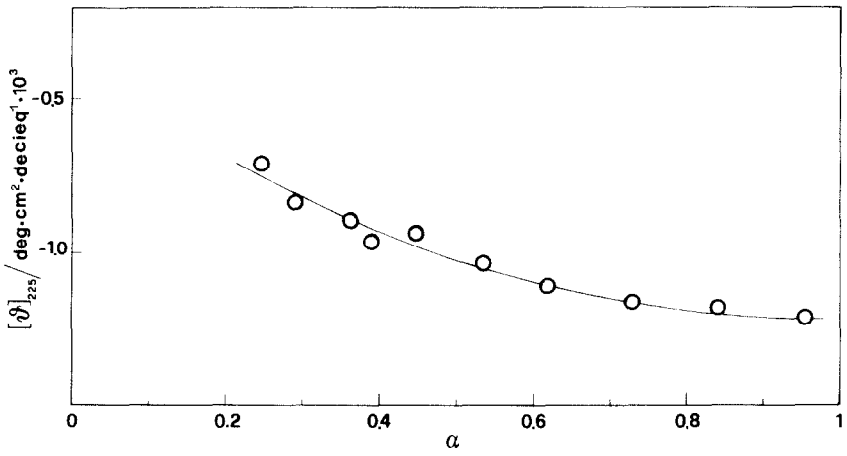


Fig. 3. Dependence of molar ellipticity ([θ]) of galactoglucan in water at 25° on the degree of ionisation (α): C 1.7×10^{-3} equiv/L.

inference can be drawn as to whether the conformation is regular and ordered, or randomly disordered.

(b) Effect of the pyruvic substituents on properties. Due to the simple structure of the galactoglucan, the presence of the pyruvic substituent may greatly affect the solution properties by conferment of charge and perturbation of the conformation. Therefore, attempts were made to prepare a depyruvated galactoglucan (PF-galactoglucan) by hydrolysis at 80° in the presence of 0.1 M $NaClO_4$ and either 2 mM $(COOH)_2$ or H_3PO_4 as the acid catalyst.

The CD signal of the chromophore at 225 nm decreased progressively and reached a plateau after 3 or 4 h in the presence of $(COOH)_2$ or H_3PO_4, respectively. A residual broad CD band remained and was ascribed mainly to acetyl groups, and to some of the chromophores of the contaminant succinoglycan. Analysis of the product revealed that ~5% of the pyruvic groups were still present. However, the trend of the intensity of the CD band was not monotonic as a function of time, as expected for a progressive hydrolysis reaction. A sharp sigmoidal change appeared after 1–2 h of "induction" time and has been related to some modification of the dissymmetric environment of the pyruvic chromophores, suggesting that the PF-galactoglucan and the galactoglucan have different conformations.

Attempts to prepare a PF-galactoglucan in larger quantities resulted in a solid material which was insoluble even in hot water. Thus, even if the native material is a random disordered polymer, the depyruvated polysaccharide could assume a regular conformation which nucleates to form insoluble (microcrystalline?) aggregates.

(c) Temperature dependence of viscosity and circular dichroism. The temperature dependence of the specific viscosity of salt-free aqueous solutions of YE-2(S1) is

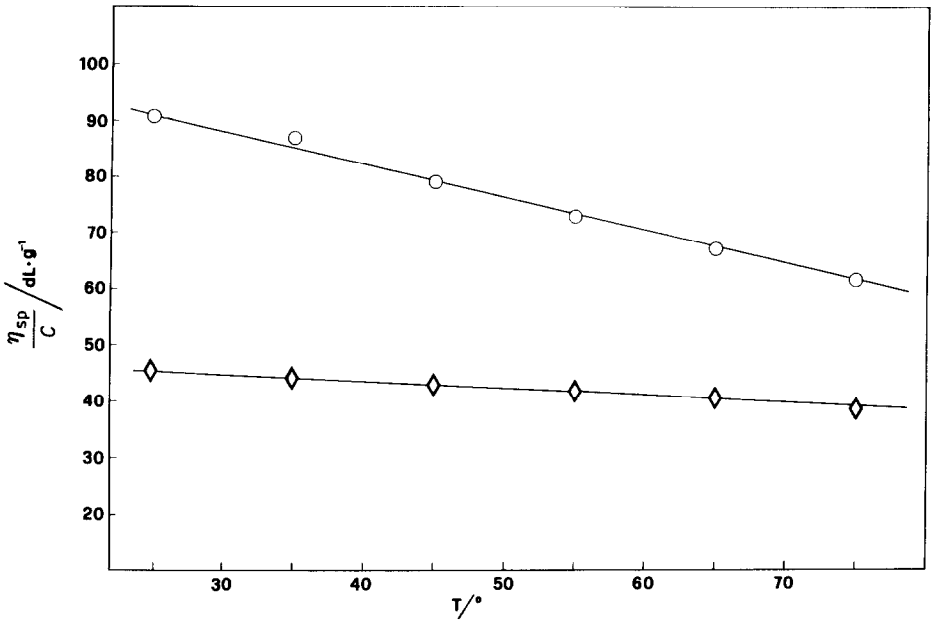


Fig. 4. Dependence on temperature of the reduced specific viscosity in water for galactoglucan of high (○) and low (◇) molecular weight.

reported as η_{sp}/C in Fig. 4 for samples of high (A) and low molecular weight (B) at concentrations of 0.12 and 0.17 g/L, respectively.

There was a negative linear trend of viscosity with increasing temperature and a higher absolute value of the slope for sample A. A similar linear trend (data not reported) was observed for a solution of the polysaccharide in 0.1 M NaCl, which, as expected, gave significantly lower values of the viscosity. The absence of a temperature-induced conformational transition is evident, in addition to the decreasing viscosity of the polysaccharide as a function of temperature, resulting possibly from the increasing randomness of an already disordered chain. This interpretation is consistent with the relative behaviour of the two samples and with the values of intrinsic viscosity, reported below.

Similarly, no evidence of a conformational transition was obtained on studying the temperature dependence of the intensity of the CD band. As shown in Fig. 5, the intensity changes in an almost linear way for sample 00-5d, whereas the sigmoidal trend, typical of the conformational transition, is exhibited by sample 04-7d, which is rich in succinoglycan. However, it could not be ascertained whether the smooth decrease (in absolute value) of the CD band was due to the galactoglucan only or, at least partially, to the contaminant succinoglycan as well.

For the purpose of our study, it is concluded that the conformation of the galactoglucan in dilute solution does not change with the temperature and is not ordered.

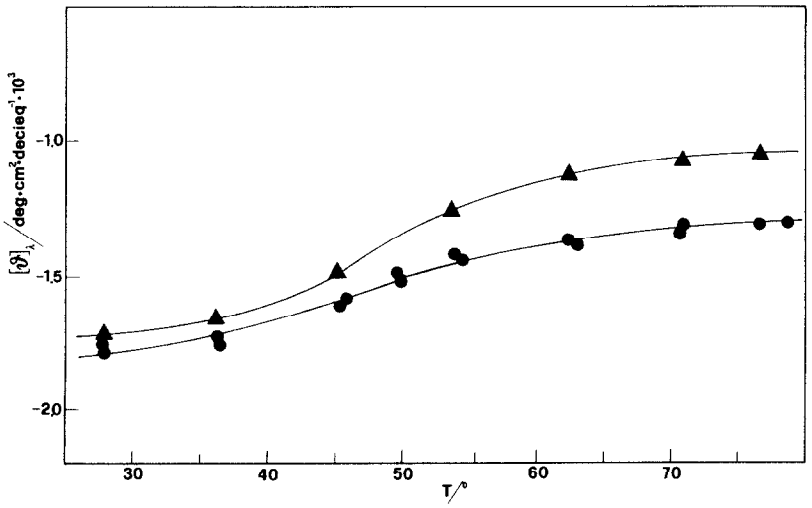


Fig. 5. Temperature dependence of the molar ellipticity ([θ]) of galactoglucan sample 00-5d (●, λ 225 nm) and of the succinoglycan-rich fraction 04-7d (▲, λ 210 nm).

(d) Hydrodynamic dimensions and chain stiffness of the galactoglucan polyelectrolyte. The viscosities of galactoglucan solutions were measured at 25° as a function of concentration, in order to calculate the intrinsic viscosity [η] and the Huggins' constant K'. Measurements were performed at several ionic strengths in the range 0.001–1 M in order to obtain the dependence of [η] as a function of the ionic strength. Table I records the results of the viscosity measurements at 0.1 M ionic strength, together with the molecular weights determined by gel-permeation chromatography. The dimensionless characteristic ratio (C_n) *in a good solvent* may be evaluated approximately from the data in Table I using [η] as a measure of the coil dimensions of the polysaccharide with eq. 5,

$$C_n = \left([\eta]/\phi M_v^{1/2}\right)^{2/3}\left(M_u/\alpha^2 L^2\right) \qquad (5)$$

where $\phi = 2.1 \times 10^{23}$ is the viscosity parameter of the Flory–Fox equation (when [η] is expressed in cm^3/g), M_v is the viscosity average molecular weight, M_u is the molecular weight of the sugar unit, and L is the virtual bond vector spanning each sugar residue ($L = 0.415$ nm). However, for practical purposes, we wish simply to compare the "perturbed characteristic ratio" of the two polysaccharide samples as a function of the ionic strength. This choice is due to lack of information regarding the value of the second virial coefficient, which is needed in order to evaluate the expansion factor α^2. The results of these calculations are reported in the last entries in Table I and show substantial agreement between the dependence on the ionic strength of the characteristic ratio C_n for the two samples with different molecular weights. The fully screened chain in a good solvent exhibits overall

TABLE I

Hydrodynamic dimensions of galactoglucan samples

I/M [a]	$[\eta]$ (g/cm^3) [b]	Rg (nm) [c]	$\langle r^2\rangle^{1/2}$ (nm) [c]	C_n [c]
A. Mol wt 267×10^3, dp 580				
1	426	13.6	81.5	33.2
0.25	457	13.9	83.4	34.8
0.15	480	14.1	84.8	36.0
0.10	488	14.2	85.3	36.4
∞	364	12.8	77.1	29.7
B Mol wt 777×10^3, dp 1689				
0.50	645	22.3	133.6	30.7
0.25	720	23.1	138.6	33.0
0.10	821	24.1	144.8	36.1
∞	520	20.7	124.0	26.6
C Theoretical value: $C_\infty = 20$ [d]				

[a] Ionic strength. [b] Intrinsic viscosity. [c] Radius of gyration, mean end-to-end distance, and characteristic ratio of the galactoglucan calculated from intrinsic viscosity with equations *4* and *5*. [d] Unperturbed characteristic ratio calculated from molecular mechanics.

dimensions about twice those calculated for the unperturbed statistical coiled chain from molecular mechanics (see below).

The viscosity data as a function of the ionic strength provide other useful information, since the elastic forces (of conformational and statistical origin) are counter-balanced to a variable extent by the repulsive electrostatic forces[23]. As expected for a polyelectrolyte in the absence of a co-operative conformational transition, a linear dependence of $[\eta]$ upon $I^{-0.5}$ is observed for samples of both high and low molecular weight (Fig. 6), with the slope diminishing with the mass of the polysaccharide.

According to Smidsrød and Haug[23], the chain stiffness can be estimated from the normalised slope (S) of the $[\eta]$ vs. $I^{-0.5}$ plot following the equation:

$$S = B([\eta]_{0.1})^{\nu}$$

where B is the Smidsrød empirical parameter and $[\eta]_{0.1}$ is the intrinsic viscosity of the polymer at $I = 0.1$. Values of 0.062 and 0.043 were found for the samples of high and low molecular weight, respectively. These values give the chain the characteristic of moderately hindered flexibility, comparable to that of carboxymethylcellulose with ds < 1 and alginate with a copolymeric composition of ~ 60% of mannuronate.

(e) Heats of dilution. The enthalpy of dilution was measured over a suitable range of concentration in order to calculate the enthalpy change ($\Delta_{dil}H$) from an initial arbitrary concentration (C_0) of 2.2×10^{-2} equiv/L to the final concentration (C). The results reported in Fig. 7 as a function of log C show a negative trend, as predicted by the polyelectrolyte theory. The increasingly higher error bars are due not only to the fact that heat changes measured at low concentration are

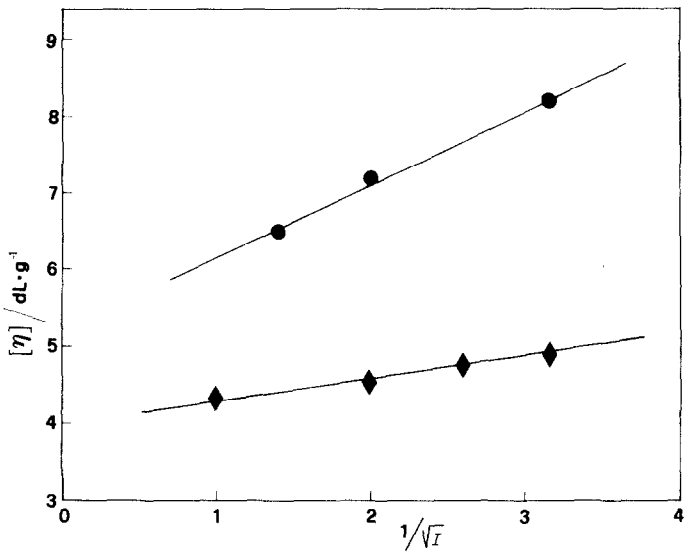


Fig. 6. Dependence of the intrinsic viscosity on the reciprocal square root of the ionic strength for galactoglucans of high (●) and low (◇) molecular weight.

vanishingly small, but also because each enthalpy value is an integral heat summed over all steps from C_0.

Provided that, upon dilution, the conformational features of the polyelectrolyte do not change significantly at the local level, the slope of the curves should

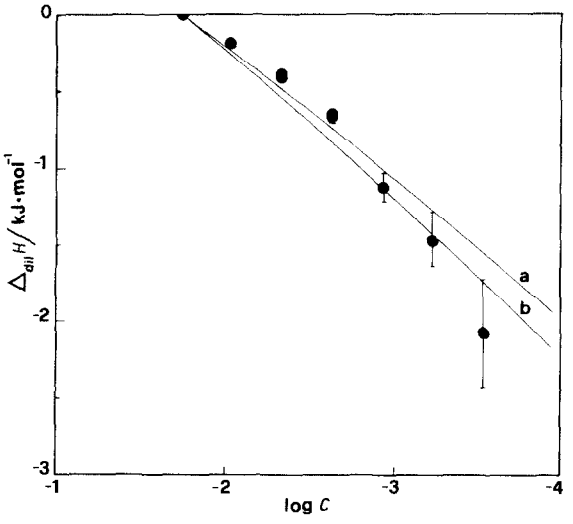


Fig. 7. Dependence of the enthalpy of dilution as a function of log C; theoretical curves calculated according to eq. 2 for ξ values of 0.89 (a) and 0.98 (b).

depend, according to eq. *3*, only on the value of ξ. This "thermodynamically"-derived structural parameter is an average value over the conformational fluctuations of the chain in the dissolved state.

The experimental data reported in Fig. 7 are compared with two theoretical lines. One line was obtained by using a structural charge parameter (ξ) of 0.89, calculated from the regular (helical) conformation of the polysaccharides, as deduced from X-ray diffraction studies[24]. The other line was obtained by using a value of $\langle\xi\rangle$ of 0.98, calculated from the average value of the extension of short chains following the conformational calculations reported here. The agreement is satisfactory for each chain model, which differ mainly with regard to the rigidity of the local conformation. However, where a regular helical structure is not preserved in solution upon dilution, the heat of dilution shows large positive deviations[25]. The present agreement indicates, on the one hand, that unscreening the polyelectrolyte does not change substantially local-chain extension (i.e., of the order of 3–5 nm), and, on the other, that the theoretical approach does not suffer from excessive idealisation of the model.

Conformational analysis.—(a) Conformational energy surfaces of the dimeric unit. As outlined in the theoretical section, the aim of this part of the investigation was not only to calculate theoretically the unperturbed dimensions of the galactoglucan random chains and the extension of the chain at short distances, but also to understand the influence of pyruvic substitution on the conformation.

The minimum energy conformation of 3-*O*-[4,6-*O*-(1-carboxyethylidene)-α-D-galactopyranosyl]-β-D-glucopyranose has been obtained by an iterative procedure, giving the structure reported in Fig. 8. The conformational energy surfaces of the two dimeric segments α-Gal-β-Glc and β-Glc-α-Gal, with and without the pyruvic substituent, were evaluated (Figs. 9 and 10) with a grid of 10°. The glycosidic bond in α-Gal is axial and in β-Glc it is equatorial.

The presence of the pyruvic substituent does not affect the overall contour pattern markedly, although comparison of Figs. 9a and 10a, and Figs. 9b and 10b, indicates a reduction of conformational accessible space (for states with $E < 1$ kcal/mol) and also a shift of the population of conformers. However, it is impossible from these qualitative results to reach conclusions about the influence

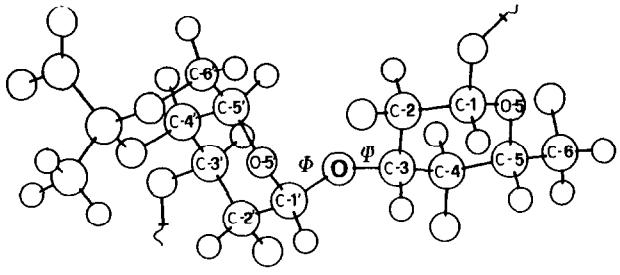


Fig. 8. Minimum-energy conformation of the disaccharide unit of the galactoglucan.

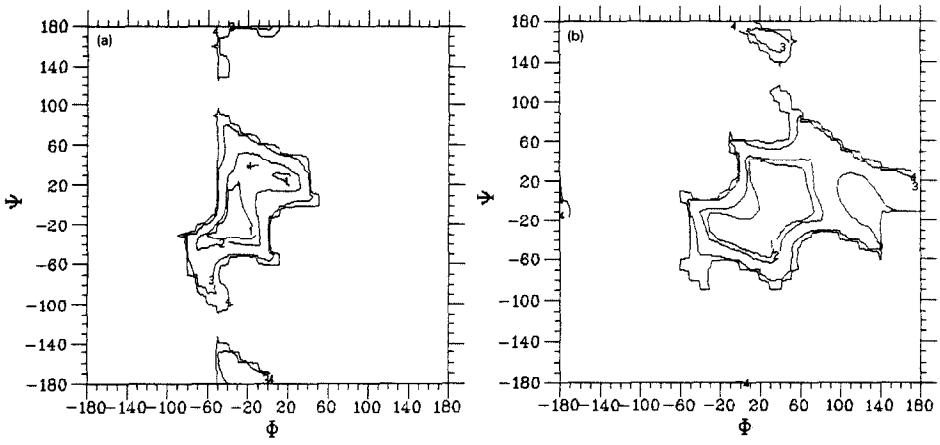


Fig. 9. Conformational energy surfaces of the α-Gal-β-Glc (a) and β-Glc-α-Gal (b) residues without the pyruvic substituent.

of pyruvate substituents on the conformationally dependent properties of the polysaccharide. In fact, whereas the reduction of the accessible conformational surface is closely related to the configurational entropy, topological features depend on the purely geometrical arrangement of the consecutive virtual bonds.

(b) Chain conformation and dimensions. The overall configurational features of the galactoglucan chain are better described by the plots of Fig. 11 and by the value of C_∞ reported in Table I. Fig. 11 shows the trend of the characteristic ratio

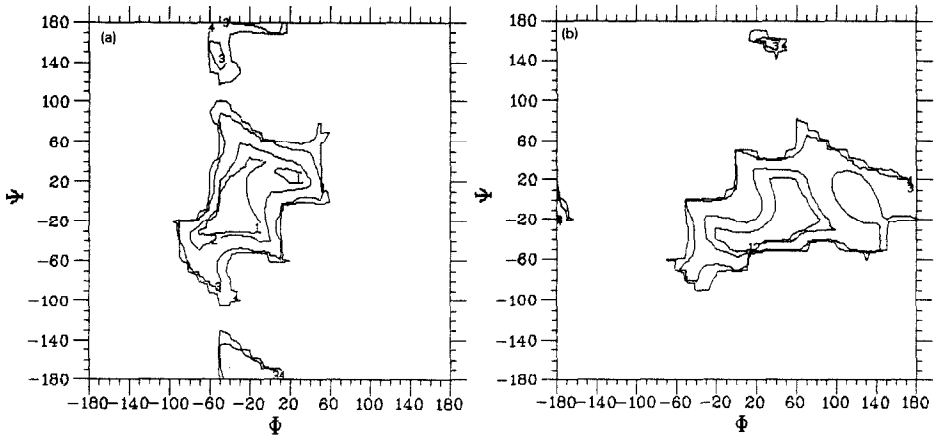


Fig. 10. Conformational energy surfaces of the α-Gal-β-Glc (a) and β-Glc-α-Gal (b) residues with the pyruvic substituent.

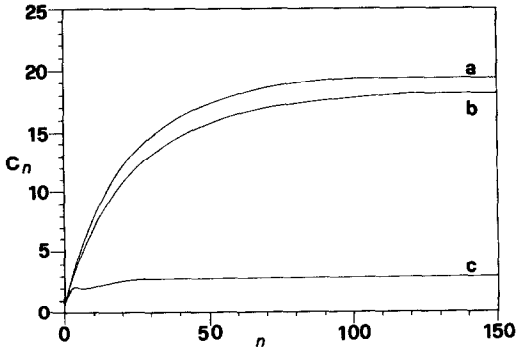


Fig. 11. Chain-length dependence of the characteristic ratio of the (1 → 3)-α-D-galactan (a), the (1 → 3)-β-D-glucan (c), and the galactoglucan with the pyruvic substituent (b).

as a function of the number (n) of residues for galactoglucan and for the two cognate homopolysaccharides. The behaviour of the (1 → 3)-β-D-glucan has been reported[26] to be that of a very flexible and loosely helical conformation with a C_∞ value of 3.1. The behaviour of the other homopolysaccharide, (1 → 3)-α-D-galactan, is expected to be similar to that of a (1 → 3)-α-D-glucan. In fact, a value of 21.7 has been evaluated for the characteristic ratio of this galactan. Contrary to expectations, the characteristic ratio C_n of the galactoglucan is not an average of these two constituent units. This result reflects the alternating sequence of the two units, since the characteristic ratio is a function of the statistics of the sequence for alginate[27].

It is also instructive to compare the correlation function for the three polysaccharides (Fig. 12). This function provides an average description of the chain trajectory and is particularly effective for the persistence of the topological orientation of the virtual bonds. The striking oscillatory function of the glucan and the more inflexible, extended function of the galactan coalesce in the zigzag decay

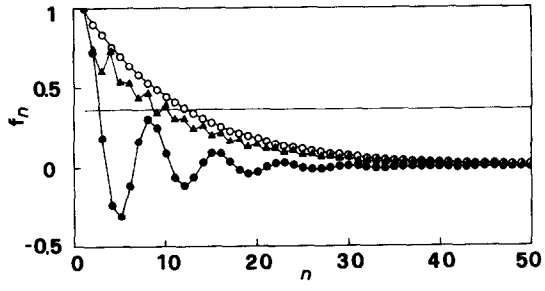


Fig. 12. Chain-length dependence of the bond correlation function f_n of the (1 → 3)-α-D-galactan (○), the (1 → 3)-β-D-glucan (●), and the galactoglucan with the pyruvic substituent (▲).

observed for the galactoglucan, which is a consequence of the alternating sequence.

CONCLUSIONS

Thus, the solution properties clearly show that the galactoglucan from *Rhizobium meliloti* mutant YE-2(S1) does not assume a regular conformation under the normal range of physicochemical conditions. The relatively simple chemical constitution makes it possible to compare the experimental results with those predicted by two theoretical models. The agreement is satisfactory and provides a sounder basis for the statement founded on experimental data only.

The solution properties of the galactoglucan are typical of a statistical random coil characterised by moderate flexibility. The chain is slightly more flexible than that of a $(1 \rightarrow 3)$-α-D-glucan. The local moderate stiffness gives a contour line suitable for the theoretical calculation of the counterion condensation polyelectrolyte theory. The random-coil behaviour was also demonstrated by the rheological behaviour of the galactoglucan-rich mixture, typical of an entanglement system and, moreover, comparable to that of guar. Unlike the structure of galactomannans, the regular simple structure of the galactoglucan could easily reveal periodic patterns of intra- and inter-molecular interactions leading to aggregation–dimerisation and gelling phenomena.

The absence of these phenomena, under most conditions of the present investigation, and the possible role of pyruvic and of acetate substituents have to be taken into account in the structure–properties relations. Selective modifications of the native polysaccharide are feasible and their effects on the properties should be investigated.

It is also important to take into account the physiological role of the galactoglucan component in a mixture produced under environmental control. Osmotic stresses are known to displace the biochemical pathways towards the synthesis of intracellular metabolites which effectively counterbalance the change of the water chemical potential. However, the exocellular production of polysaccharides must have other functional properties, in addition to its important role in nodulation, such as a protective action. Selective binding of ions by the gelling succinoglycan component may be one of such protection mechanisms. The symbiotic interaction would then depend not necessarily on the cation-binding properties of the polysaccharide.

ACKNOWLEDGMENTS

We thank Dr. L.P.T.M. Zevenhuizen for his collaboration, Dr. P. Faleschini for isolation of the polysaccharide, Dr. F. Zanetti for the gel-permeation chromatography, Dr. R. Urbani for computational advice, and the "Progetto Finalizzato del

CNR, Chimica Fine" and the National Research Council (CNR), Rome for financial support.

REFERENCES

1 M. Yalpani (Ed.), *Industrial Polysaccharides*, Elsevier, Amsterdam, 1987.
2 L.P.T.M. Zevenhuizen, in S.S. Stivala, V. Crescenzi, and I.C.M. Dea (Eds.), *Industrial Polysaccharides*, Gordon & Breach, New York, 1987, pp. 45–68.
3 L.P.T.M. Zevenhuizen, in E.A. Dawes (Ed.), *Novel Biodegradable Microbial Polymers*, Kluwer Academic Publishers, 1990, pp. 387–402.
4 J. Glazebrook, J.W. Reed, T.L. Reuber, and G.C. Walker, *Int. J. Biol. Macromol.*, 12 (1990) 67–70.
5 L.P.T.M. Zevenhuizen, in V. Crescenzi, I.C.M. Dea, S. Paoletti, S.S. Stivala, and I.W. Sutherland (Eds.), *Biomedical and Biotechnological Advances in Industrial Polysaccharides*, Gordon & Breach, New York, 1989, pp. 301–311.
6 L.P.T.M. Zevenhuizen and P. Faleschini, *Carbohydr. Res.*, 209 (1991) 203–209.
7 L. Navarini, A. Cesàro, and S.B. Ross-Murphy, *Carbohydr. Res.*, 223 (1992) 227–234.
8 G.S. Manning, *Acc. Chem. Res.*, 12 (1979) 443–449.
9 G.S. Manning, *Q. Rev. Biophys.*, 11 (1978) 179–246.
10 G.S. Manning, *J. Phys. Chem.*, 88 (1984) 6654–6661.
11 A. Cesàro, in H.-J. Hinz (Ed.), *Thermodynamic Data for Biochemistry and Biotechnology*, Springer, New York, 1986, pp. 177–207.
12 S. Paoletti, A. Cesàro, F. Delben, V. Crescenzi, and R. Rizzo, in P. Dubin (Ed.), *Microdomains in Polymer Solutions*, Plenum Press, New York, 1985, pp. 159–189.
13 A. Cesàro and J.C. Benegas, *Makromol. Chem. Rapid Commun.*, 10 (1989) 547–552.
14 A. Cesàro, S. Paoletti, R. Urbani, and J.C. Benegas, *Int. J. Biol. Macromol.*, 11 (1989) 66–72.
15 D.A. Brant, *Q. Rev. Biophys.*, 9 (1976) 527–596.
16 R. Urbani and A. Cesàro, *Polymer*, 32 (1991) 3013–3020.
17 S. Arnott and W.E.J. Scott, *J. Chem. Soc., Perkin Trans. 2*, (1972) 324–335.
18 A. Hybl, R.E. Rundle, and D.E.J. Williams, *J. Am. Chem. Soc.*, 87 (1965) 2779–2788.
19 P.J. Garegg, P.-E. Jansson, B. Lindberg, F. Linh, J. Lönngren, I. Kvarnström, and W. Nimmich, *Carbohydr. Res.*, 78 (1980) 127–132.
20 P.J. Flory, *Statistical Mechanics of Chain Molecules*, Wiley–Interscience, New York, 1969.
21 D.A. Brant and M.D. Christ, in A.D. French and J.W. Brady (Eds.), *Modeling of Carbohydrate Molecules*, American Chemical Society, Washington DC, 1990, pp. 42–68.
22 A. Cesàro and M.C. Sagui, *Makromol. Chem., Makromol. Symp.*, (in press).
23 O. Smidsrød and A. Haug, *Biopolymers*, 10 (1971) 1213–1227.
24 R. Chandrasekaran, personal communication.
25 A. Cesàro, S. Paoletti, and J.C. Benegas, *J. Thermal Anal.*, (submitted).
26 B.A. Burton and D.A. Brant, *Biopolymers*, 22 (1983) 1769–1792.
27 G.M. Hallman and S.G. Whittington, *Macromolecules*, 6 (1973) 386–389.