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Interaction of soluble chitosans with dyes in water. II. Thermodynamic data

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Three water-soluble chitosan derivatives, namely *N*-carboxymethyl chitosan (NCMCh), *N*-carboxybutyl chitosan (NCBCh), and the reduction product of the aldimine obtained from chitosan and 5-hydroxymethyl-2-furaldehyde (NHMFCh), were found to interact in dilute aqueous solution with anionic dyes. The binding was studied at 25°C with optical and thermodynamic (i.e. isothermal microcalorimetry and dilatometry) techniques under weakly acidic conditions. By using the Scatchard and Hill methods, the values of the binding constants were obtained from the absorption spectra. They ranged from 6×10^4 to 7×10^8 litre mol^{-1}. The binding ability seems to decrease in the order NHMFCh > NCBCh > NCMCh and Orange II > Alizarin S = Congo Red > Alizarin GG, respectively. From the ΔG° and ΔH values of binding the variation of entropy with interaction was computed for the chitosan derivatives–dyes systems. The values of ΔS of interaction were very small or negative and the ΔV of binding were nil, indicating that no desolvation of the species occurs on binding.

INTRODUCTION

Evidence for the interaction in water of some anionic dyes with chitosan and three water-soluble chitosan derivatives, namely *N*-carboxymethyl chitosan (NCMCh), *N*-carboxybutyl chitosan (NCBCh), and the reduction product of the aldimine obtained from chitosan and 5-hydroxymethyl-2-furaldehyde (NHMFCh), was collected by optical techniques. The objective of the present work was to obtain quantitative data, by improved spectroscopic methods and thermodynamic techniques (isothermal microcalorimetry and dilatometry).

MATERIALS AND METHODS

Materials

The water-soluble chitosan derivatives and the anionic dyes were the same as indicated in Part I.*

*Stefancich, S., Delben, F. & Muzzarelli, R.A.A. (1984). *Carbohydr. Polym.*, **24**, 17–23.

The *n*-heptane used as a dilatometric liquid was a C. ERBA RP product (Milan, Italy). It was purified prior to use as described elsewhere (Katz & Ferris, 1966).

Instruments and methods

The absorption spectra recorded upon addition of a polysaccharide solution to a dye solution are generally very complicated. In particular, for many systems the band(s) of the complex and the band(s) of the free dye were hardly resolved. A more sophisticated procedure, allowing the band(s) from the complex to be evidenced, was used in the case of the binding of Cu(II) and Pb(II) by chitosan derivatives, and was described in detail by Dobetti and Delben (1992). In this case the amount of non-polymeric species added was the same in the sample cell and in the reference cell. In addition, because of the higher absorption of pure dyes compared with the polymer–dye complexes, the sample cell was replaced by the two half-path-length reference cells, and vice versa. Thus, the excess free dye gave a positive absorption, while the polymer–dye complex showed negative bands.

To achieve quantitative binding data, the following

method was employed. Increasing amounts of dye solution were put into a cuvette containing a polysaccharide solution. Known aliquots of dye solution were put into the reference cuvette, containing the solvent, to offset the differential spectra of free dye. Because the concentrations of total dye, free dye and polymer are known, it is possible to construct the Scatchard plots (Scatchard, 1949) over a wide range of bound dye-to-polymer molar ratio, r.

The UV-visible spectra were recorded with a Varian Cary 2200 spectrophotometer (Varian Techtron, Mulgrave, Victoria, Australia) employing quartz cells. In the spectrophotometric measurements the polymer concentration never exceeded $1{\cdot}5 \times 10^{-3}$ M.

The calorimetric determinations were performed at 25°C with a flow-type 10700-1 LKB isothermal microcalorimeter (LKB-Produkter AB, Bromma, Sweden) equipped with gold cells. The solutions were introduced into the calorimeter with a P-3 Pharmacia peristaltic pump (Pharmacia Fine Chemicals AB, Uppsala, Sweden), equipped with Pharmacia THFE tubings. The flow rates of both the polysaccharide and the dye solutions were very similar, with a range of $5{\cdot}5$–$6{\cdot}2 \times 10^{-6}$ liter s^{-1}. In the experiments, the polymer solution was first mixed with water and then with dye solutions having increasing concentrations. With this procedure, the dilution effect of the polysaccharide was automatically compensated. The dilution heat of the dye was measured separately and subtracted. Electrical calibrations were performed occasionally. The concentration of the polymer solutions was $1{\cdot}5$–$5{\cdot}0 \times 10^{-3}$ M. The maximum value of the total dye-to-polymer molar ratio reached in the calorimetric measurements was established for each system at the beginning of the precipitation of the complex.

The dilatometric measurements were performed using Carlsberg dilatometers (Linderstrøm-Lang & Lanz, 1938), which were located in a thermostatic bath, as described by Delben (1980). In each experiment, 4 ml of polymer solution were mixed with 4 ml of dye solution. The maximum value of R reached in the dilatometric measurements was generally 0·2. The polymer concentration was $3{\cdot}5$–$4{\cdot}1 \times 10^{-3}$ M. The dilution effects of both the polysaccharide and the dye were measured separately and taken into account. n-Heptane was used as the dilatometric liquid.

The pH of the solutions was measured with a Radiometer (Copenhagen, Denmark) PHM52 pHmeter, equipped with GK2321C combined electrodes at ambient temperature (about 23°C).

RESULTS AND DISCUSSION

Some representative UV-visible absorption spectra, recorded according to the first procedure described in the previous section, are reported in Fig. 1. In most cases, the interaction with the polysaccharide induces new band(s) in the dye spectra, as shown in Figs 1(a) and (b). This stems from a strong interaction between the chromophore and the polymeric backbone or between the dye molecules lodged on the polysaccharide chain. In some other cases, quenching of the dye spectra is only observed (hypochromic effect), as shown in Fig. 1(c). This phenomenon cannot be ascribed to a lack of binding ability, and is not an artifact due to dilution since this has already been corrected for. In these cases the binding is possibly essentially electrostatic, with a minor perturbation of the chromophore. To achieve quantitative binding data from our spectra in this case, the method

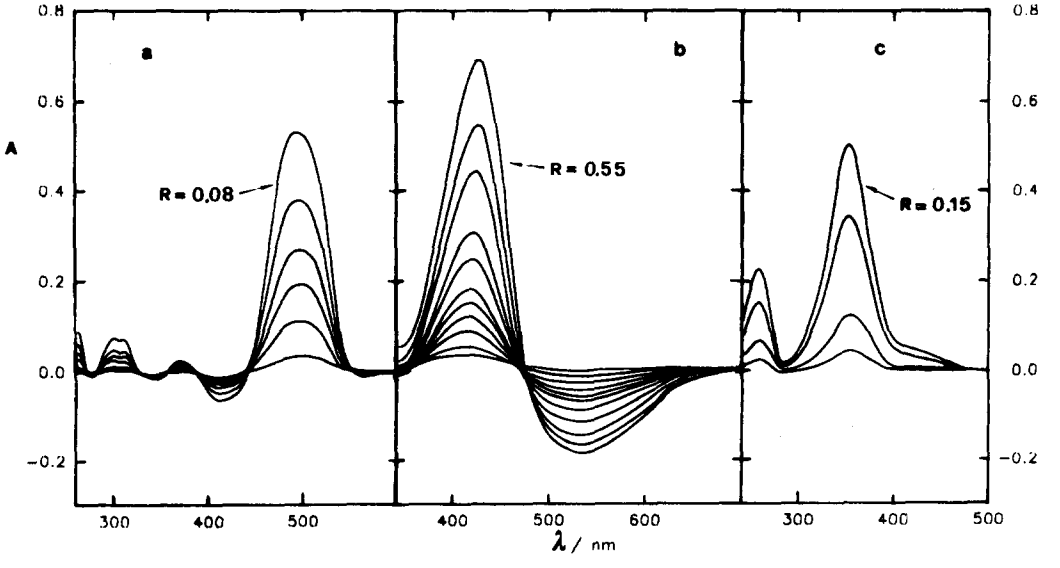

Fig. 1. Absorption spectra recorded using the twin-cuvette procedure of a, NCBCh–Orange II; b, NCBCh–Alizarin S; c, NCBCh–Alizarin GG. R is the total dye-to-polymer molar ratio.

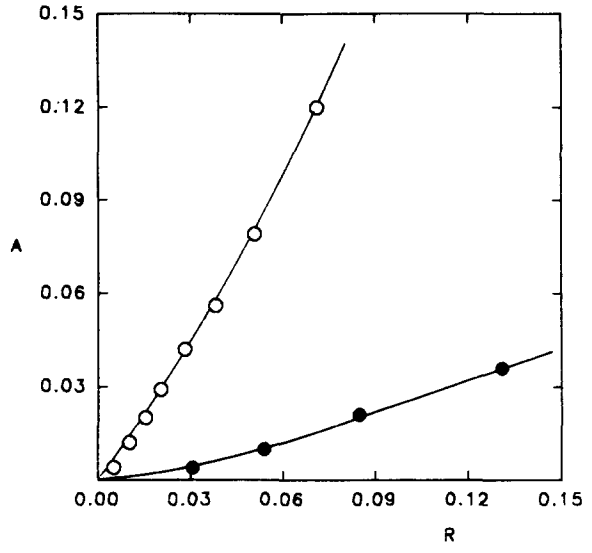

Fig. 2. Absorption of the maximum of the band of the dye–polysaccharide complex versus the total dye-to-polymer molar ratio, *R*. ○, NCBCh–Orange II; ● NHMFCh–Orange II.

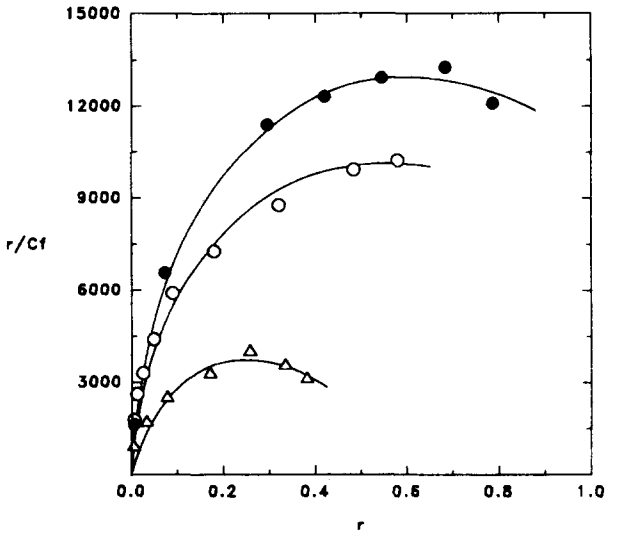

Fig. 3. Scatchard plots obtained as described in the text. ○, NCBCh–Orange II; ●, NHMFCh–Orange II; △, NHMFCh–Alizarin S. *r* denotes the ratio of bound dye to polymer; C_f is the molar concentration of free dye.

described in Part I seems to be the only possibility.

On the other hand, for the systems which permit the absorption bands of the complex to be distinguished from those of the free dye, qualitative information on the binding can be derived by plotting the intensity of one of the bands of the complex as a function of the total dye-to-polymer molar concentration, *R*. In Fig. 2 two typical cases are reported. The sigmoidal shape of the plots stems from cooperativity in both cases.

Quantitative data were derived according to the procedure described in the Instruments and Methods section, which allowed the free dye concentration, C_f, to be computed provided that the dye–polysaccharide complex does not absorb at the wavelength chosen to offset the differential spectra of the free dye, which most often corresponded to the absolute maximum in the visible spectra of the dye itself. Based on inspection of the spectra obtained with the twin-cuvette method, this condition seemed to be substantially verified. The total dye, C_t, and the polymer, C_p, concentrations being known, the well-known Scatchard plots can be drawn, since the concentration of the bound dye, C_b, is

$$C_b = C_t - C_f. \quad (1)$$

In Fig. 3 the Scatchard plots for three typical cases are reported. The relevant *r* versus C_f plots are reported in Fig. 4. From the plots of both figures, cooperativity in the binding was evident. This result was in agreement with the previous, qualitative findings (Fig. 2). Whether cooperativity is derived from a conformational transition of the polysaccharide backbone induced by the dye or from a stacking of the dyes molecules onto the polymer is a matter of speculation. In principle, both phenomena can take place simultaneously.

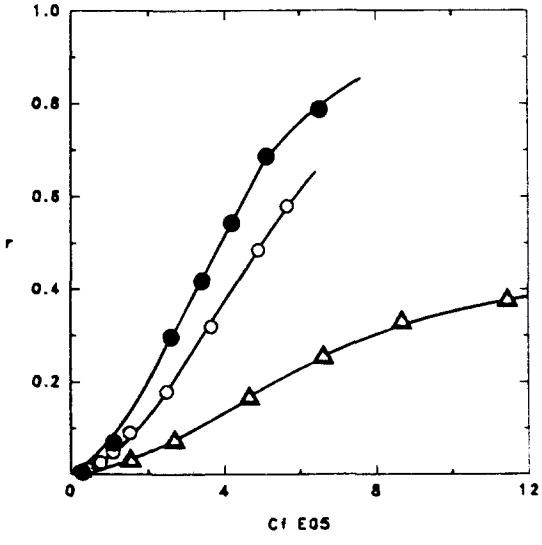

Fig. 4. Plots of *r*, the ratio of bound dye to polymer, versus C_f, the concentration of free dye. ○, NCBCh–Orange II; ●, NHMFCh–Orange II; △, NHMFCh–Alizarin S.

Many interactions of biopolymers with non-macromolecular species are characterized by cooperative binding. Chitosan derivatives and dyestuffs being of potential biological significance, the cooperativity found here seems very meaningful.

In the case of cooperative binding, the overall stability constant K_B is not simply derived from the Scatchard plots, since it is a function of *r*. In order to correlate the results obtained with the various systems, we computed the values of K_B by using the semi-empirical approach usually employed in these cases. This approach is based on the assumption that the binding over part of the saturation range can be described by

equations phenomenologically resembling those for an infinitely cooperative system (Cantor & Schimmel, 1980). The 'all-or-none' reaction which represents the binding in the case of an infinite cooperativity leads to the following equation:

$$r/n = (C_f^n/K^n)/(1 + C_f^n/K^n) \quad (2)$$

where K is the apparent dissociation constant for the interacting sites and n is the number of dye molecules which saturate the binding site(s) onto the polymer.

If a partially cooperative binding is considered, as it is in real cases, n is replaced by the Hill constant $\alpha(H)$ (Hill, 1910), which is an index to the cooperativity:

$$r/n = (C_f^{\alpha(H)}/K^{\alpha(H)})/(1 + C_f^{\alpha(H)}/K^{\alpha(H)}). \quad (3)$$

The Hill constant can be derived from eqn (3):

$$\alpha(H) = d\{\ln[r/(n-r)]\}/d(\ln C_f). \quad (4)$$

Thus, the Hill constant can be obtained from the slope of a plot of $\ln[r/(n-r)]$ versus $\ln C_f$ (Hill plot). For $\alpha(H) > 1$, the Scatchard plot passes through the origin of the axes (r/C_f and r, respectively). At low values of r or C_f, the curve rises and reaches a maximum (r_{max}) at

$$r_{max} = n[\alpha(H) - 1]/\alpha(H). \quad (5)$$

At higher values of r, the curve drops to intersect the r-axis at $r = n$.

Finally, the following equation is obtained:

$$\ln C_f = -[1/\alpha(H)]\ln[n/r - 1] + \ln K. \quad (6)$$

Equation (6) allows the K-value to be computed from the Hill plot. Then, the overall binding constant can be obtained:

$$K_B = C_b/C_f^{\alpha(H)}(nC_p - C_b) = [K^{\alpha(H)}]^{-1}. \quad (7)$$

The values of all the experimental parameters, reported in Table 1, deserve some comments. First, the values of $\alpha(H)$ can be obtained from both the Hill plots, as the slope of the linear portion of the curve (eqn (4) or eqn (6)), and from eqn (5). In both cases, the value of n, which is obtained from the Scatchard plots at the value of r at which the curve intercepts the r-axis, is of crucial importance. In our cases, n was determined from the Scatchard plots for a few systems only. For the other systems, this simple determination was not possible because the experimental data points were limited to the first portion of the Scatchard plot: i.e. lower values of r. Then, the values of n were chosen to obtain the best linearity in the Hill plots. A typical example of these plots is shown in Fig. 5. In turn, eqn (5) was employed to compare the experimental values of r_{max} with the values computed from $\alpha(H)$ and n-values (by eqn (5)). The good agreement generally found confirmed that the choice of the values of n was substantially correct.

Secondly the values of n ranged from 0·6 to 1·2. In most instances, n was equal to 1, a surprisingly high

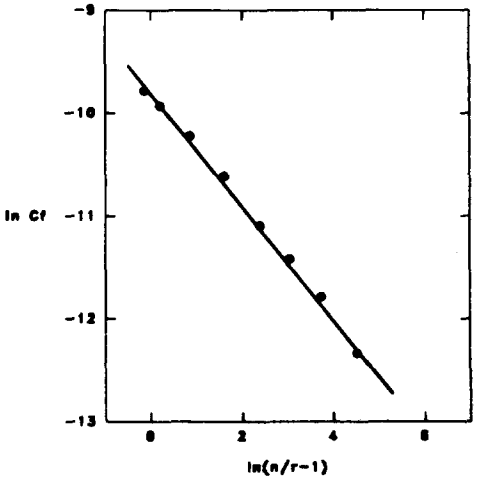

Fig. 5. Hill plot for the NCBCh–Orange II system.

Table 1. Thermodynamic results (for symbols, see text)

System	pH	K^a (mol liter^{-1})	$\alpha(H)$	n	r_{max} eqn (5)[b]	r_{max} Scatchard[c]	$K_B = 1/K^{\alpha(H)}$ (liter mol^{-1})	$\Delta G°$ (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (e.u.)
NCMCh–Orange II	3·5	—	—	—	—	—	—	—	(−24)[d]	—
NCMCh–Alizarin S	3·5	$4·53 \times 10^{-4}$	1·43	0·8	0·24	(0·2)	$6·0 \times 10^4$	−6·5	−49·0	−143
NCMCh–Alizarin GG	5·0	—	—	—	—	—	—	—	(−13)[d]	—
NCBCh–Orange II	3·5	$5·71 \times 10^{-5}$	1·88	1·1	0·51	(0·8)	$9·5 \times 10^7$	−10·9	−11·1	−1
NCBCh–Alizarin S	3·5	$1·79 \times 10^{-4}$	1·53	1·0	0·35	(0·4)	$5·0 \times 10^5$	−7·8	−14·7	−23
NCBCh–Alizarin GG	5·0	$3·10 \times 10^{-4}$	1·48	1·0	0·32	(0·5)	$1·0 \times 10^5$	−7·1	−18·9	−40
NHMFCh–Orange II	3·5	$4·50 \times 10^{-5}$	2·04	1·2	0·61	0·65	$7·0 \times 10^8$	−12·1	−10·0	7
NHMFCh–Alizarin S	3·5	$7·88 \times 10^{-5}$	1·79	0·62	0·27	0·26	$2·2 \times 10^7$	−10·0	−15·5	−18
NHMFCh–Alizarin GG	5·0	$2·89 \times 10^{-4}$	1·72	0·6	0·25	0·14	$1·2 \times 10^6$	−8·3	−34·0	−86
NHMFCh–Congo Red	3·5	$4·54 \times 10^{-5}$	1·64	1·0	0·39	(0·4)	$1·3 \times 10^7$	−9·7	—	—

[a]Microscopic dissociation constant.
[b]Values from eqn (5).
[c]Values from the Scatchard plots.
[d]Approximate values; see text.

value, indicative of a very extensive binding of the dyes onto the polymer, because $n = 1$ means that at saturation one dye molecule is bound to one saccharide repeat unit. In fact, the polymer concentrations were calculated from the average molecular weight of the saccharidic units, taking into account the proportions of *N*-acetyl glucosamine, glucosamine and *N*-alkyl glucosamine. This is in agreement with the results of Maghami and Roberts (1988), who demonstrated a 1:1 stoichiometry in the binding of sulfonate dyes by chitosan. The binding of divalent metal cations by the same chitosan derivatives showed very different features: the *n*-values found in that case being much lower, about 0·1 (Delben *et al.*, 1989; Dobetti & Delben, 1992). In other words, the polysaccharides are able to bind an amount of anionic dyes (expressed in moles) an order of magnitude larger than metal cations. This seems to indicate that, while metal cations are bound onto specific binding sites (Delben & Muzzarelli, 1989; Delben *et al.*, 1992; Dobetti & Delben, 1992), the dye molecules interact with a larger portion of the polymer chain. Moreover, the sign of the charge localized onto the low-molecular-weight species is crucial.

Finally, from the K_B-values the binding ability can be deduced for both the polysaccharides and the dyes studied. The experimental errors were not computed because of the difficulty in evaluating the uncertainties in the experimental parameters. However, assuming that they were small enough to allow at least the order of magnitude of the binding constants to be exactly determined, the binding ability seemed to decrease in the order NHMFCh > NCBCh > NCMCh, and Orange II > Alizarin S = Congo Red > Alizarin GG, respectively.

The results of the calorimetric analysis are reported in Fig. 6 as enthalpy variation, normalized by the total polysaccharide repeat units, as a function of *r*. The plots were mostly linear and had negative slopes, showing that the binding is exothermic in all cases investigated. Only the NCMCh–Alizarin GG system showed a detectable cooperativity, in qualitative agreement with the general spectroscopic evidence.

The slope of the linear portion of the curve gives the enthalpy change upon binding, normalized per mole of bound dye (Table 1). For the NCMCh–Orange II and NCMCh–Alizarin GG systems the ΔH values reported are approximate, because for these systems, giving only a hypochromic effect in the absorption spectra, the amount of bound dye was not computed with confidence (see Part I). The negative and surprisingly large ΔH values mostly lay in the range −10 to −20 kcal mol^{-1} bound dye. In the case of the NCMCh–Alizarin S and NHMFCh–Alizarin GG systems, values of −49 and −34 kcal mol^{-1} bound dye, respectively, were found. These values seem to indicate a very strong and extended interaction between the dyes and the polysaccharides. Moreover, with the notable exception of

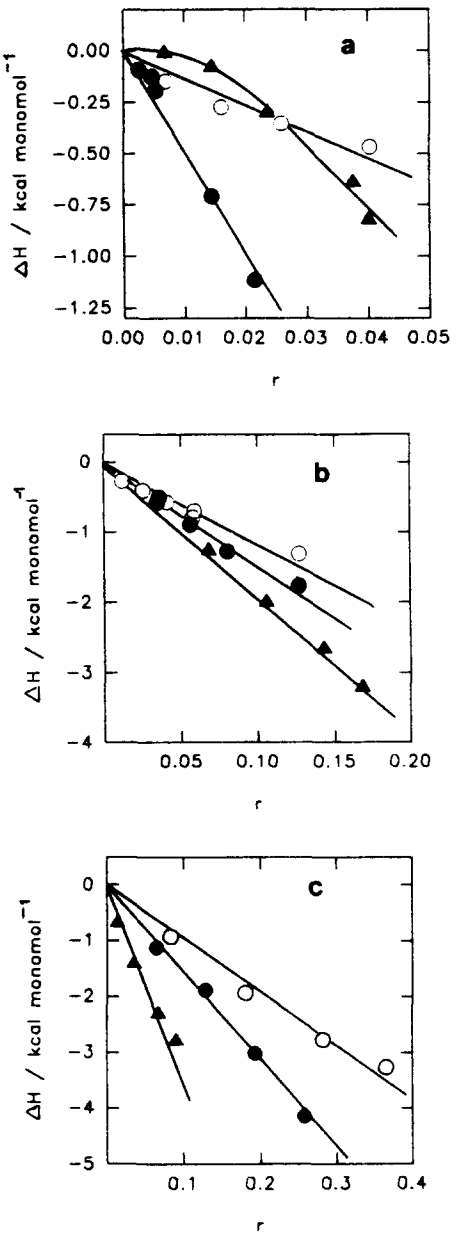

Fig. 6. Calorimetric results for the systems studied. a, NCMCh; b, NCBCh; c, NHMFCh. ○, Orange II; ●, Alizarin S; ▲, Alizarin GG.

the NCMCh–Alizarin S and NHMFCh–Alizarin GG systems, the binding seems to be very similar in nature in all the systems investigated.

The dilatometric measurements were performed at two *R* values (i.e. 0·1 and 0·2) for each system. The experimental data were properly corrected by the dilution effects of both the polysaccharide and the dye solutions. They were found to be 0 ± 2 ml mol^{-1} of dye (± SD) in all cases.

The ΔV of binding are usually related to the ΔS values, which can be computed from ΔG° (where $\Delta G^\circ = -RT \ln K_B$) and ΔH, assuming that ΔG and ΔG° are not very different. The ΔS values were found

to be mostly very small or negative. The ΔS and the ΔV values seemed to contradict the calorimetric results. In fact, small or nil (or even negative) ΔS and ΔV values should indicate that no desolvation of the species occurs on binding. One must conclude therefore that the dyes are extensively bound onto the polysaccharides studied, but that the hydration spheres of the interacting species are not destroyed by the interaction. Indeed, in the case of a strong desolvation of interfering species, positive ΔV of binding should be expected, similar to what happens in the case of cation–polycarboxylate systems (Paoletti *et al.*, 1981; Crescenzi *et al.*, 1974; Delben & Paoletti, 1974; Cesàro *et al.*, 1988; Delben & Muzzarelli, 1989).

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REFERENCES

Cantor, C.R. & Schimmel, P.R. (1980). *Biophysical Chemistry.* W.H. Freeman & Co., San Francisco, pp. 849–86.

Cesàro, A., Delben, F. & Paoletti, S. (1988). *J. Chem. Soc., Faraday Trans., 1,* **84,** 2573.

Crescenzi, V., Delben, F., Paoletti, S. & Škerjanc, J. (1974). *J. Phys. Chem.,* **78,** 607.

Delben, F. (1980). *La Chim. & l'Ind.,* **62,** 398.

Delben, F. & Muzzarelli, R.A.A. (1989). *Carbohydr. Polym.,* **11,** 221.

Delben, F. & Paoletti, S. (1974). *J. Phys. Chem.,* **78,** 1486.

Delben, F., Muzzarelli, R.A.A. & Terbojevich, M. (1989). *Carbohydr. Polym.,* **11,** 205.

Delben, F., Stefancich, S. & Muzzarelli, R.A.A. (1992). *Carbohydr. Polym.,* **19,** 17.

Dobetti, L. & Delben, F. (1992). *Carbohydr. Polym.,* **18,** 273.

Hill, A.V. (1910). *J. Physiol.,* **40,** iv.

Katz, S. & Ferris, T.G. (1966). *Biochemistry,* **5,** 3246.

Linderstrøm-Lang, K. & Lanz, H. (1938). *Comp. Rend. Trav. Lab. Carlsberg, Ser. Chim.,* **21,** 315.

Maghami, G.G. & Roberts, G.A.F. (1988). *Makromol. Chem.,* **189,** 2239.

Paoletti, S., Cesàro, A., Ciana, A., Delben, F., Manzini, G. & Crescenzi, V. (1981). In *Solution Properties of Polysaccharides,* ed. D.A. Brant. ACS Symp. Series, No. 150, American Chemical Society, Washington, DC, p. 379.

Scatchard, G. (1949). *Ann. N.Y. Acad. Sci.,* **51,** 660.