

Interaction of soluble chitosans with dyes in water.

I. Optical evidence

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The interaction of several cationic and anionic dyes with *N*-carboxymethyl chitosan (NCMCh), *N*-carboxybutyl chitosan (NCBCh), the reduction product of the aldimine obtained from chitosan and 5-hydroxymethyl-2-furaldehyde (NHMFCh) and the parent chitosan was studied in water with optical techniques. The interaction with cationic dyes was studied in the pH range of 5–6. No interaction was observed. The anionic dyes were able to modify the spectra of soluble chitosans in the pH range 2–5, the most remarkable alteration appearing at pH 3–5. For all the polysaccharide–dye systems studied, absorption and circular dichroism spectra were collected at different dye-to-polymer molar ratio values.

INTRODUCTION

The study of the interaction between macromolecules and low-molecular-weight species in solution and in the solid state is of great importance from the theoretical and practical points of view. The binding in solution of small molecules and ions by biopolymers has been of interest for many years, because these complexes provide an insight into general biomacromolecular interactions with substrates. The absorption of dyestuffs and other chemicals by solid polymers has been widely investigated, mainly for industrial applications.

At least 70 dyes (mainly anionic and direct dyes) have so far been studied in terms of their adsorption on chitin/chitosan. Direct dyes were used by Tokunaga (1963) in coloring chitosan-treated cotton fabrics. Acridine Orange, a diarylmethane dye, was used for the study of polyelectrolyte complexes by Cundall *et al.* (1979). Highly polymerized dyes based on chitosan and other polymers were made by Ciba S.A. (1962). The dye-binding capacity of solid chitin and, extensively, of deacetylated chitosan as a function of pH was studied by Knorr (1983). The equilibrium sorption of Methyl Orange, an anionic dye, by chitosan and its octanoyl,

dodecanoyl and octadecanoyl derivatives was investigated by Seo *et al.* (1988, 1989), who explored the hydrophobic domains formed by cationic polymers; the amounts of dye sorbed were five times larger than for the parent chitosan. Equilibrium data were presented by McKay (1987) and analysed using the Langmuir, Freundlich and general isotherm expressions: capacities were in the range 40–200 mg dye g⁻¹ chitin. Several azo dyes were studied by Giles *et al.* (1958), who pointed out the physical and electrostatic nature of the interaction, and by Yamamoto (1984) and Yamamoto *et al.* (1984), who described their chiral interaction with chitosan. Maghami and Roberts (1988) demonstrated a 1:1 stoichiometry between protonated amino groups in the chitosans and sulfonic acid groups in the dye ions. Recently, Pal and Pal (1990) found that the stoichiometry of interaction of some dyes with cationic chitosan is one dye at every alternate cationic site of the polymer.

Most of the studies in the literature have been carried out with regard to their applicability in the textile and food industries and do not actually contain refined thermodynamic and analytical data. In the present work, we have approached the problem of the interactions of dyestuffs with modified water-soluble chitosans

in terms of analytical chemistry and thermodynamics of dilute solutions at various pH values.

MATERIALS AND METHODS

Materials

The modified chitosans *N*-carboxymethyl chitosan (NCMCh), *N*-carboxybutyl chitosan (NCBCh) and the reduction product of the aldimine obtained from chitosan and 5-hydroxy-2-furaldehyde (NHMFCh) were prepared according to the procedures described by Muzzarelli *et al.* (1982, 1989) and Delben *et al.* (1992), respectively. The data concerning the polysaccharides are given in Table 1.

The concentrations of the polymers were calculated from the amount of the freeze-dried polysaccharide dissolved and the volume of the resulting solution. The amount of water in the lyophilized solid was taken into account. It was 20.5, 14.4, 18.4 and 15.7% for NCMCh, NCBCh, NHMFCh and chitosan, respectively. The values of the average molecular weights of the repeat units of the polysaccharides are also reported in Table 1.

The anionic dyes considered in this study were Orange II (4-[(2-hydroxy-1-naphthalenyl)azo] benzenesulfonic acid monosodium salt), 95% pure; Alizarin S (9,10-dihydro-3,4-dihydroxy-9,10-dioxo-2-anthracene-sulfonic acid monosodium salt), 75% pure; Alizarin GG (2-hydroxy-5-[(3-nitrophenyl)azo]benzoic acid monosodium salt), 50% pure; Congo Red (3,3'-[(1,1'-biphenyl)-4,4'-diylbis-(azo)]bis(4-amino-1-naphthalene-sulfonic acid) disodium salt). All were supplied by Aldrich Chemical Co. (St Louis, MO, USA).

Alizarin GG was crystallized from ethanol; the final purity was computed to be 75% by spectrophotometry.

The following cationic dyes were also tested: Neocuproin hydrochloride (2,9-dimethyl-1,10-phenanthroline hydrochloride), from Riedel-de Haën AG (Seelze, Germany); 1-(2-pyridilazo)-2-naphthol, from Merck (Darmstadt, Germany); Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) and Ruthenium Red (ruthenium oxychloride ammo-

niated), both from Sigma Chemical Co (St. Louis, MO, USA).

The dialysis membranes were cellulose tubings (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany). Prior to use, these tubings were treated as described elsewhere (Crescenzi *et al.*, 1974).

Perchloric acid for pH adjustments of the polymer and dye solutions was obtained by diluting 70% HClO₄ to 0.1 M. Deionized, doubly-distilled water was constantly used.

Instruments and methods

The fluorescence spectra were recorded with a Hitachi MPF-3 (Hitachi Ltd., Tokyo, Japan) or with a Jasco PP-770 (Japan Spectroscopic Co. Ltd., Tokyo) spectrofluorimeter. Because of the qualitative character of the fluorescence analysis, neither the correction for self-absorption was performed nor the quantum yield of the spectrofluorimeter checked.

The circular dichroism (CD) measurements were performed with a Jasco J600 dichrograph (Japan Spectroscopic Co. Ltd.) or with a Jasco J500-A dichrograph equipped with a Jasco DP 500-N data processor. Four spectra were usually cumulated for each reading. Both the spectrophotometer and the dichrograph were equipped with a thermostatic unit. The temperature was measured inside the sample cuvette with a HD 8464 DeltaOhm (Padua, Italy) electronic thermometer.

The UV-absorption spectra were recorded with a Varian Cary spectrophotometer (Varian Techtron, Mulgrave, Victoria, Australia) as the difference between dye-containing and dye-free solutions after introduction in both sample and reference cells of aliquots of polymer solution and thorough stirring.

In the UV-absorption and CD measurements the molar polymer concentration, C_p , ranged from 3 to 8×10^{-4} monomol liter⁻¹.

The pH of the solutions was determined with a Radiometer (Copenhagen, Denmark) pHM52 pHmeter, equipped with GK2321C and GK2421C combined electrodes, or with a Radiometer pHM85 pHmeter, equipped with GK2401 combined electrodes.

The polymer solutions were prepared as described

Table 1. Properties of chitosan and chitosan derivatives

Name	Parent chitosan		Molecular weight	Degree of acetylation	Degree of <i>n</i> -substitution	Water average (%) ^a	M_w of the repeat unit ^b
	Source	Supplier					
Chitosan	<i>Euphausia superba</i>	Rybex	465 000	0.42	0	15.7	186.9
NCMCh	<i>Euphausia superba</i>	Rybex	465 000	0.42	0.58	20.5	212.5
NCBCh	<i>Chionoecetes opilio</i>	Katakura	628 000	0.15	0.35	14.4	202.5
NHMFCh	<i>King crab</i>	Protan	441 000	0.15	0.30	18.4	200.5

^aWater content in the freeze-dried polysaccharide.

^bValues used to compute the concentrations of polymers. In the case of chitosan, the presence of acetate ions in the freeze-dried solid was taken into account.

elsewhere (Dobetti & Delben, 1992). The concentration was calculated from the volume of the resulting solution, taking into account the water contained in the lyophilized polysaccharide, previously determined with a Mettler DL 18 Apparatus (Mettler Instruments AG, Greifensee, Switzerland), employing the Karl-Fischer method.

RESULTS AND DISCUSSION

The fluorescence spectra of the dyes, both in the absence and in the presence of the polysaccharides, were generally too weak to be used as a probe of the interaction.

The anionic dyes studied were found to interact with the polysaccharides, by using CD measurements. In particular, in the presence of soluble chitosans they exhibited a CD band, whose intensity (and, in a few cases, shape) was pH-dependent. Hypothesizing that the height of this band indicates the extent or the strength of the binding (Dobetti & Delben, 1992), the CD spectra of all the systems under study were recorded at fixed R values, the molar ratio of dye to polysaccharide repeat units (R 0.1) and at pH values ranging from 6 to about 2.5.

Mostly, the maximum height in the CD spectra appeared around pH 3.5, which was adopted therefore as the pH for the subsequent work. In the case of Alizarin GG, as the CD spectra were only slightly different at pH 3.5 and 5, the latter pH value was chosen for the subsequent measurements to avoid precipitation of the dye, which possesses a carboxylic group instead of a sulfonic one as the acidic function.

The CD spectra of three dye-polysaccharide systems at different pH values are reported in Figs 1–3 as sample cases. Particularly interesting, in Fig. 1 an inversion of the CD band upon decreasing pH is observed, which indicates that pH can affect not only the extent of binding but also the mode of binding. This inversion in the CD band could not be explained properly; however, it can be excluded that this phenomenon is due to a conformational variation of the chitosan chain with pH. In fact, on the basis of thermodynamic and CD measurements it was found that the chitosan backbone should remain in an expanded conformation, regardless of the pH (Delben *et al.*, 1989).

A precise comparison of the present results with literature CD data (Yamamoto, 1984) is impossible, because in the cited paper the degree of deacetylation of the polysaccharide is not specified. However, it can be underlined that in the case of Congo Red the CD spectra (not shown) are qualitatively coincident with those found by Yamamoto (1984). On the contrary, the CD spectra of the chitosan–Alizarin GG system are different in terms of wavelength of the isodichroic point and sign of both CD bands exhibited by the complex (Fig. 2).

At pH 3.5 (or pH 5 in the case of Alizarin GG) the

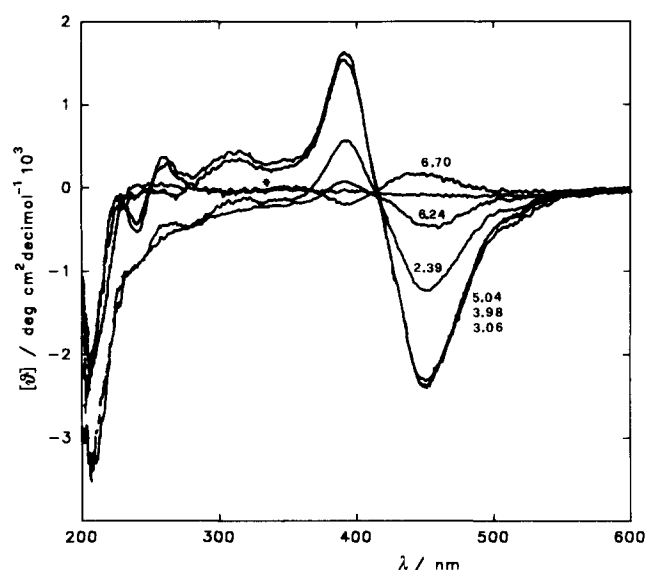


Fig. 1. CD spectra of *, chitosan at pH 6.1 and of the chitosan–Orange II system at $R = 0.12$ and at different pH values (quoted). Polymer concentration, 4.1×10^{-4} M. R denotes the molar ratio of dye to polysaccharide repeat units.

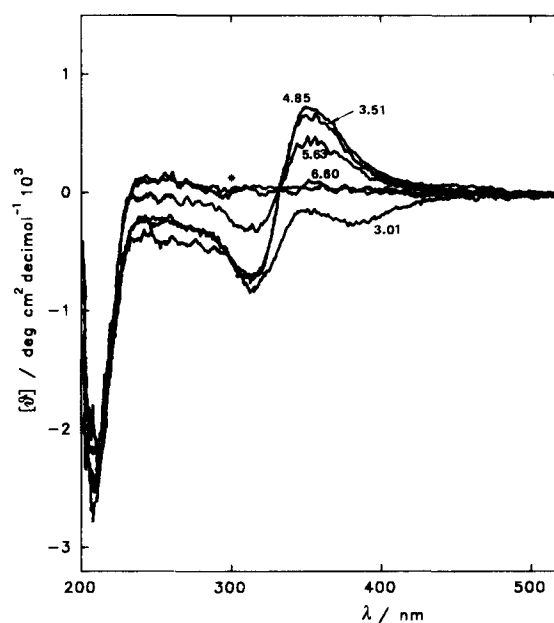


Fig. 2. CD spectra of *, chitosan at pH 6.1 and of the chitosan–Alizarin GG system at $R = 0.13$ and at different pH values (quoted). Polymer concentration, 3.9×10^{-4} M. R denotes the molar ratio of dye to polysaccharide repeat units.

UV-visible absorption spectra were recorded as a function of the molar ratio R for all the systems under study. Increasing amounts of the polymer solution were added into a cuvette containing a known volume of dye solution. The corresponding spectra, recorded against a dye-free polymer solution, were reported in Fig. 4 for Alizarin S, Alizarin GG and Orange II in the presence of NCBCCh. Very similar spectra were obtained in the presence of NCMCh or NHMFCh (unreported).

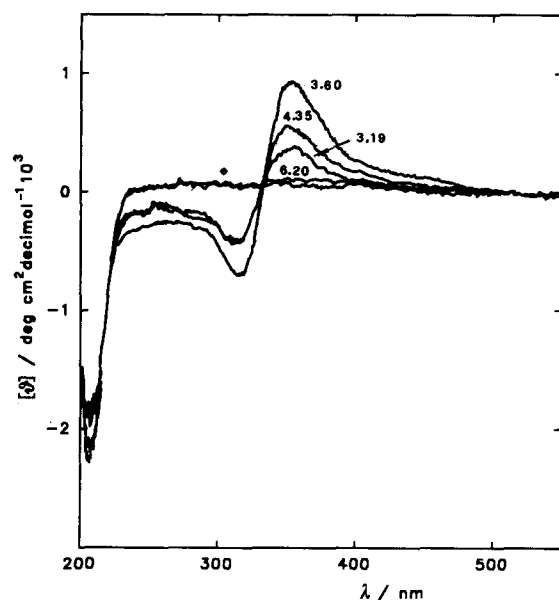


Fig. 3. CD spectra of *, NCBCh at pH 6.0 and of the NCBCh-Alizarin GG system at $R = 0.10$ and at different pH values (quoted). Polymer concentration, 5.1×10^{-4} M. R denotes the molar ratio of dye to polysaccharide repeat units.

The spectra appeared somewhat complicated; however, some common features were detectable. First, in all the cases studied the absorption of the band(s) of the pure dye was decreased on addition of polymer solution, and the variation was significantly higher than for the dilution alone. Secondly, the polymer mostly induced the presence of new band(s), so confirming in these cases the presence of a binding of the dyes by the polysaccharides.

Quantitative data of binding were tentatively calcu-

lated from these spectra for a few dye-polymer systems. In all cases, however, in the Scatchard (1949) plots the derived data points were scattered to such an extent that it was hard to compute even the order of magnitude of the binding constants.

An attempt to collect quantitative binding data with equilibrium dialysis experiments in order to refine these results failed because of the very large affinity of the dyes towards the cellulose tubings used. Therefore, to confirm the uncertain results obtained new spectrophotometric experiments were prepared, as mentioned in Part II* of this study. However, for the systems in which the addition of polymer solution to dye solution had only a hypochromic effect, without inducing new bands, the method here employed to elaborate the spectra appeared the only one possible to obtain quantitative, although approximate, data from spectroscopic measurements — this being the case for NCMCh-Orange II and for NCMCh-Alizarin GG systems.

For the systems which exhibited the sharpest CD bands related to the dye-polysaccharide complex, spectra were collected as a function of the ratio R at pH 3.5 or, in the case of Alizarin GG, at pH 5 to avoid precipitation of the dye at higher concentrations. Owing to the high absorption of the dye, the noise in the spectra was too high to allow a fine analysis of the bands. However, interesting, albeit qualitative, indications were obtained. Only a few cases will be discussed for simplicity.

The addition of Orange II to a modified chitosan solution induced the formation of two well-pronounced

*Stefancich, S., Delben, F. & Muzzarelli, R.A.A. (1994). *Carbohydr. Polym.*, **24x**, 25–30.

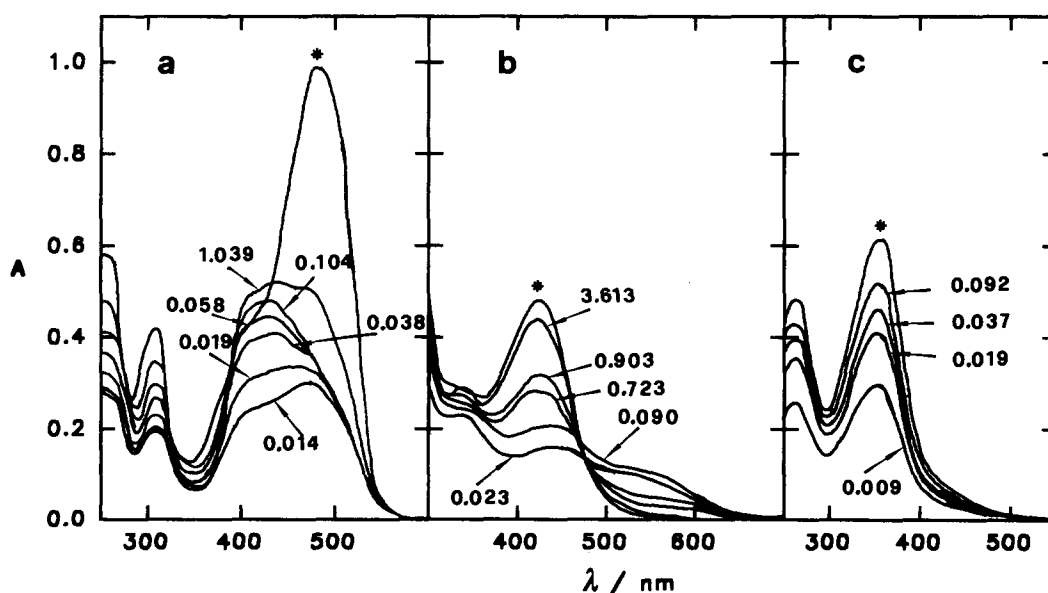


Fig. 4. Absorption spectra of (a) Orange II, (b) Alizarin S and (c) Alizarin GG in the absence (*) and in the presence of increasing amounts of NCBCh at pH 3.5. Values of R , the molar ratio of dye to polysaccharide repeat units are quoted.

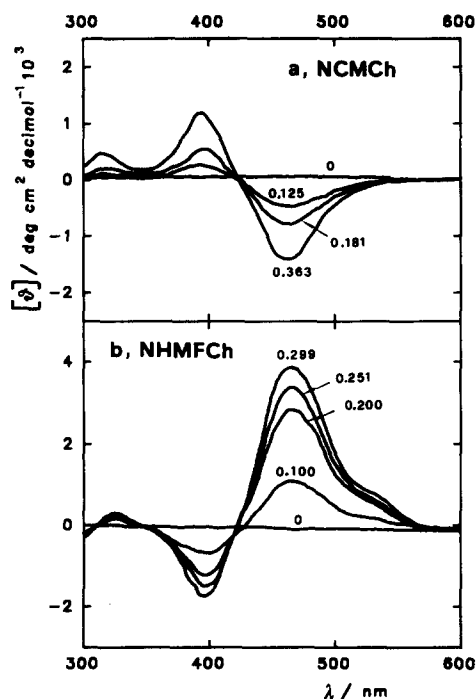


Fig. 5. CD spectra of (a) NCMCh and (b) NHMFCh in the presence of increasing amounts of Orange II at pH 3.5. The polymer concentration was 4×10^{-4} and 3×10^{-4} M, respectively. Values of R , the molar ratio of dye to polysaccharide repeat units are quoted.

bands with an isodichroic point at 420 nm. With NHMFCh, these bands were opposite in sign with respect to the other polysaccharides; this parallels the fact that the band at 225 nm, typical for the NHMFCh, was positive rather than negative as usually found for the other chitosan derivatives and chitosan itself (Fig. 5). This evidence confirmed previous findings (Delben *et al.*, 1992) that the derivatization operated with S5-hydroxymethyl-2-furaldehyde changed the chirality of the chitosan backbone. Moreover, in the NCMCh–Orange II system a few weak bands in the near-UV region indicated that the dye molecules extensively perturb the polymer backbone.

Even more complicated CD spectra are presented by Alizarin S complexes. In Fig. 6 the CD spectra of the NCMCh–Alizarin S and NHMFCh–Alizarin S systems are reported. In the former case, the polymer–dye complex was characterized by two CD bands — a negative band centered around 244 nm and a positive one centered around 265 nm. An isodichroic point between these bands was clearly detectable. Moreover, the negative band centered around 210 nm, typical for NCMCh, was increasingly but slightly perturbed by the presence of the dye. In the latter case, two main characteristics are worth noting. First, upon addition of Alizarin S new dichroic bands appeared in the spectrum — a negative band centered around 269 nm and a positive one centered around 251 nm. It is remarkable that in the case of the NCMCh–Alizarin S system the nega-

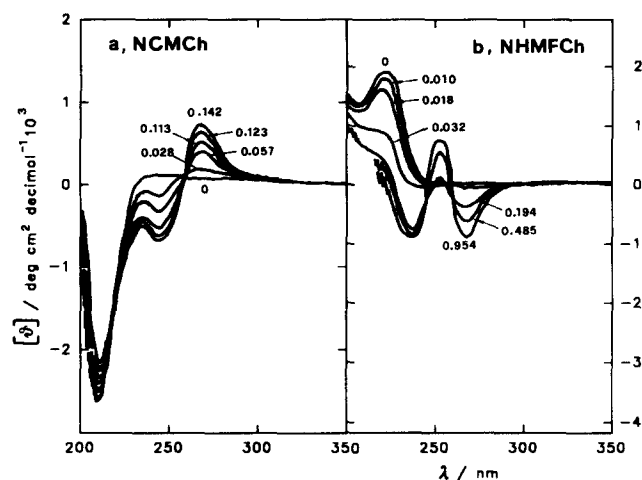


Fig. 6. CD spectra of (a) NCMCh and (b) NHMFCh in the presence of increasing amounts of Alizarin S at pH 3.5. The polymer concentration was 4×10^{-4} and 3×10^{-4} M, respectively. Values of R , the molar ratio of dye to polysaccharide repeat units are quoted.

tive peak appeared at lower wavelength values, while in the case of the NHMFCh–Alizarin S system the negative peak was exhibited at higher wavelength values than the positive one. Second, the peak around 225 nm, which is typical for NHMFCh, was strongly affected by the dye. The positive peak decreased upon addition of Alizarin S and, at the same time, a new negative band at 235 nm appeared. At R values higher than 0.2 the negative band only was detectable. It is a matter of speculation whether this feature indicates a change in the chirality of the polymer backbone. One can only be sure that in this case the dichroic spectra showed a very intense engagement in the binding of the polymer side-chain, which is responsible for the positive band of the polysaccharide in the absence of dye.

Conversely, in the other systems studied a precise indication of the site of binding was not given by the spectra so far recorded. Indeed, in general the CD spectra of dye-soluble chitosan systems are less complicated than the spectra shown and discussed here, and a weaker perturbation of the polysaccharide chain upon binding could be proposed. Moreover, since the CD spectra were typical for each system, it could be concluded that, with the notable exception of a few cases, the binding of anionic dyes by chitosan derivatives is aspecific, in the sense that the binding site should involve a wide portion of the polymer chain and the side groups as well.

Among the anionic dyes employed in this study, Congo Red was found to behave in a very peculiar way. Its aqueous solutions are red at pH values above 5 and blue below this limit. The turn from the red to the blue form at pH 3.5 was slow and followed the trend shown in Fig. 7, which indicates cooperativity. This is surprising, if we consider that the two forms only differ in that

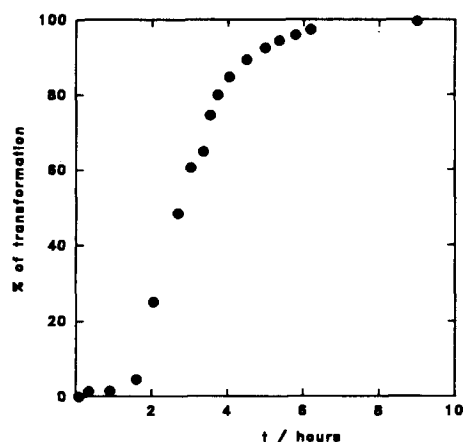


Fig. 7. Kinetics of transformation of a solution of Congo Red at pH 3.5 from the red to the blue form expressed as transformation percentage as a function of time. Dye concentration, 2.3×10^{-5} M.

new resonance structures are possible in the blue form (Finar, 1963). This behavior seriously hindered the absorption measurements, because the spectra were dependent on both molar ratio R and time. However, evidence of strong interaction of Congo Red with the three chitosan derivatives was collected. The spectra of the polysaccharide-dye systems significantly differed from the spectra of the dyes alone, both in the red and in the blue forms. In Fig. 8 a sample case was reported. With NCMCh, moreover, a tentative dialysis equilibrium experiment showed that at pH 3.5 a blue-coloured polymer-free solution was in equilibrium with a red-coloured polymer-containing solution, producing a sensational effect. It is uncommon to find solutions so different in color, in spite of their membrane equilibrium. The value of R in this experiment was 0.22.

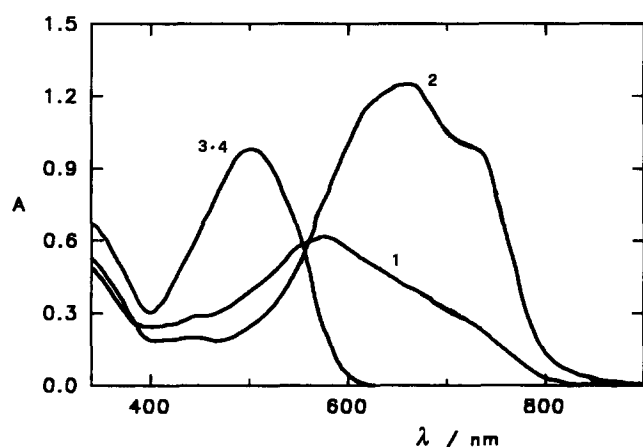


Fig. 8. Absorption spectra of Congo Red at pH 3.5 in the absence and in the presence of NHMFCh at R (molar ratio of dye to polysaccharide repeat units) = 0.13. 1 and 2, dye alone, immediately after and 24 h after the preparation of the solution, respectively; 3 and 4, in the presence of NHMFCh both immediately after and 24 h after the preparation of the solution, respectively.

In the case of cationic dyes, the interaction was studied in the pH range of 5–6. At the same pH values, some metal cations were found to interact with soluble chitosans (Delben *et al.*, 1992, and reference quoted therein). Higher pH values were not exploited, to prevent the insolubilization of the reacting species or the formation of weak gels. Lower pH values (down to 3) were occasionally tested, and the experimental results were exactly the same as in the pH range of 5–6. In these conditions, in the absorption spectra no variation was observed upon addition of polysaccharide solution to a dye solution at pH values ranging from 5 to 6. Only in the case of Neocuproin was a small variation upon addition of polymer detected, but the phenomenon was attributed to a small variation of pH upon addition of polymer, since this dye was very sensitive to pH.

In the case of Ruthenium Red the occurrence of interaction was also tested following the kinetics of air oxidation of the dye at different pH values in the absence and in the presence of added polysaccharide. These further measurements were performed since it is known that addition of polysaccharides which interact with Ruthenium Red in some cases only moderately affects the general shape and intensity of the absorption spectra of the dye (Murano *et al.*, 1990). On the other hand, the fading of dyes in solution is known to be slowed down in the presence of interacting polyelectrolytes (Delben *et al.*, 1976).

With the systems under study, no difference between polymer-containing and polymer-free solutions was detected, thus confirming that Ruthenium Red is not bound by chitin derivatives, analogously to the other cationic dyes. The reason can be probably ascribed mainly to the stiffness of the chitin-like polysaccharide backbone (Luft, 1971).

CONCLUSIONS

The occurrence of an interaction in water between the hydrophilic chitosan derivatives NCMCh, NCBCCh and NHMFCh and the parent chitosan and the anionic dyes Orange II, Alizarin S, Alizarin GG and Congo Red was proved by absorption spectrometry and circular dichroism measurements. The binding was found to depend on pH: the pH range 3.5–5 being the most effective in this regard. For a particular system, the pH was found to change both the extent and the mode of the binding. While the general shapes of the absorption spectra depended mainly on the dye, the CD spectra varied for each particular system studied. The latter technique was thus believed to be more powerful in order to get qualitative indications of the dye-polymer interaction.

On the contrary, the cationic dyes Neocuproin, 1-(2-pyridilazo)-2-naphthol, Ethidium bromide and Ruthenium Red were found not to interact at all with the

polysaccharides studied. The study was performed systematically in the pH range of 5–6.

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