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# Induced circular dichroism study of the aqueous solution complexation of cello-oligosaccharides and related polysaccharides with aromatic dyes

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**Abstract**

*Acetobacter xylinum*, grown in the presence of low levels of the water-soluble dye Calcofluor White ST produces a pellicle of cellulose that has no detectable crystallinity. Biological factors of this sort are probably more important than physical factors in controlling the higher order structures of celluloses. Circular dichroism (CD) is induced by complexes that are formed by specific interactions between chiral oligosaccharides and dye molecules. Using CD, equilibrium constants were measured for the association reactions between various dyes with a series of cello-oligosaccharides ($n=2$–6), methylcellulose, hydroxypropylcellulose (HPC), amylose, cyclomalto-oligosaccharides (cyclodextrins), and the linear malto-oligosaccharides ($n=3$–7). Possible structural features of the complexes are discussed. Dyes that are capable of binding to the higher cello-oligomers in aqueous solutions are the same dyes that modify the solid structure of bacterial cellulose. An analogy between the binding of water-soluble dyes to cello-oligosaccharides and the binding of the cellulose-degrading enzyme, cellobiohydrolase I, to cellulose is discussed.

*Keywords*: Oligosaccharide; Cello-oligosaccharide; Polysaccharide; Cyclomalto-oligosaccharide; Dyestuff; Oligo- and poly-saccharide complex; Circular dichroism

## 1. Introduction

Cellulose (**1**) is the principal component of plant cell walls and is probably the most abundant organic material in nature. Therefore, it is somewhat surprising that, despite a body of work dating from a century ago to the present, there remain a number of fundamental unresolved problems regarding the biosynthesis and higher order structures of cellulose

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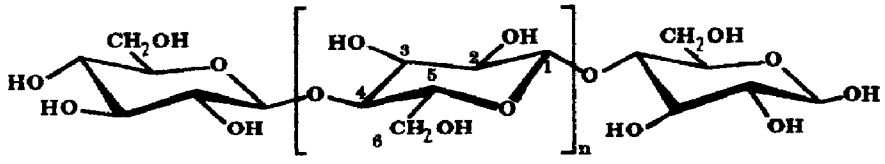
Table 1
Dyes investigated as potential ligands [CAS registry no.]

| Active Dyes | Inactive dyes |
|---|---|
| Calcofluor White (**9**) [4404-43-7] | Nile Blue A (**16**) [3625-57-8] |
| Congo Red (**10**) [573-58-0] | Light green SF yellowish (**17**) [5141-20-8] |
| Direct Orange No. 8 (**11**) [2429-79-0] | Ethyl Red (**18**) [76058-33-8] |
| Direct Yellow No. 1 (**12**) [6472-91-9] | 2,6-Dichloroindophenol (**19**) [620-45-1] |
| Direct Violet No. 1 (**13**) [2586-60-9] | Anthraquinone Blue (**20**) [12619-59-9] |
| Trypan Blue (**14**) [72-57-1] | 3,3'-Diethyloxadicarbocyanine iodide (**21**) [14806-50-9] |
| Titan Yellow (**15**) [1829-00-1] | Acridine Orange (**22**) [494-38-2] |

[1,2]. The Gram-negative bacterium, *Acetobacter xylinum*, which produces an extracellular pellicle of highly pure and crystalline cellulose, is the most commonly used model system for research on the biogenesis and morphology of cellulose [3–9].



**1, n ≥ 50 (cellulose);**

**2, n = 0 (cellobiose);**

**3, n = 1 (cellotriose);**

**4, n = 2 (cellotetraose);**

**5, n = 3 (cellopentaose);**

**6, n = 4 (cellohexaose);**

**7, n = 5 (celloheptaose);**

**8, n = 6 (cellooctaose).**

In 1980, Haigler et al. [10] reported the surprising observation that *A. xylinum*, grown in the presence of low levels of the water-soluble fluorescent dye, Calcofluor White ST (**9**), produces a pellicle of cellulose having no detectable crystallinity. This result was taken as evidence that the crystalline structure of *A. xylinum* cellulose arises from a cell-directed coordination of synthesizing sites. Subsequent research has demonstrated a similar effect of dye **9** on cellulose synthesis in the green alga, *Oocystis solitaria* [11]. Haigler and co-workers have described a series of water-soluble dyes **9–15** which are also active to various degrees in modifying the crystallinity of *A. xylinum* cellulose (Table 1). Dyes of different structural types, **16–22**, are not active [12] (Table 1) from which **16** and **17** were reported by Haigler and co-workers.

X-ray [13–15] and solid-state $^{13}C$ NMR [16] structural analyses have been done for Calcofluor-modified bacterial cellulose. Results are confusing, with X-ray lines attributed to cellulose by one author being assigned to incorporated dye aggregrates by others [17]. Because of the importance of understanding the degree to which the higher order structures of celluloses are controlled by biological rather than by physical factors, the study of chemically altered bacterial and algal celluloses remains an active field [18].

All of the work discussed above has dealt with the interaction of specific dyes, generally 9 and 10, with solid cellulose. Little attention has been given to the question of whether the dye–cellulose interactions are specific to only bulk, insoluble cellulose, or whether dye binding would also occur with water-soluble oligomeric cellulose. Wood reported small shifts in the absorption spectra of dye solutions in the presence of certain oligo- and polysaccharides [19], but stated that "cello-oligosaccharides showed little or no interaction" [20]. Ritcey and Gray [21] interpreted the origination of circular dichroism (CD) spectra for solutions of Congo Red in the presence of methylcellulose and the **5** and **6** oligomers of cellulose as evidence for molecular association. There was no CD evidence for complexation with oligomers shorter than **5**. The authors concluded that the source of the extrinsic CD is with the helical asymmetry imposed on the electronic transitions in the chromophores by their association with the sugar. There is a wealth of supporting evidence for this kind of interaction producing induced CD activity from studies of interactions of dyes and small drug molecules with polypeptides and proteins [22]. Whether a helical structure for polysaccharide molecules in solution has been proved by experiment is a subject of some debate in the literature. Vacuum-UV and CD spectra for underivatized saccharides appear to be some of the best evidence for accepting this interpretation [23,24], which is confirmed to some degree by Yalpani [25], who has provided evidence for right-handed helical structures for amyloses and extended flat ribbon structures for $\beta$ glucans.

CD spectropolarimetry is most easily understood if it is thought of as a modified form of absorbance spectrophotometry in which *differences* in the absorbances between two coincident circularly polarized beams of light that pass simultaneously through the sample are measured as a function of wavelength. The necessary molecular property that distinguishes CD from absorbance is the existence of optical activity (chirality) in analytes that are nonracemic. The union of absorbance with chirality adds leverage to the analytical selectivity of absorbance measurements, in that only when both properties are present can a molecule be CD active. Large biological macromolecules are all chiral, many are CD active, and the range of applications in the study of natural products is very wide. Proteins, polynucleotides, and carbohydrates are intrinsically CD active; however, the chromophores absorb in the UV and for some, carbohydrates in particular, only in the far-UV. In spite of the extreme difficulties that are inherent to making CD measurements in that spectral range, a great deal of work has been done in which CD data have been used to interpret the secondary and tertiary structures of these macromolecules in aqueous solutions [23,24].

In still other applications the chirality inherent to these same large molecules has been exploited in experiments where activity was induced into CD inactive molecules by either substitution or molecular complexation reactions. For the carbohydrates, for example, CD spectra that originate from exciton coupling between absorbing aromatic substituents have been used to interpret the anomeric forms of linkages and the local stereochemistry between neighboring diols in monomeric repeat units [26,27]. Complexations of iodine and 1-

butanol with amylose [28] are reactions that are typical of the second type. Molecular association reactions are most familiar, and especially those for small molecules that are able to fit into the receptive hydrophobic chiral interiors of the cyclodextrin oligomers [29].

The special advantage of binding an achiral dye molecule to a chiral substrate is that the extrinsic CD activity is observed in the experimentally convenient and readily accessible visible range. The conformational rearrangement of the dye, as it adapts to the chiral field imposed upon it by association with the carbohydrate molecule, will generate an extrinsic CD spectrum that will reveal information about the structure of the host in solution in a way that is quite analogous to the application of the exciton coupling models described in the previous paragraph. Explained another way, structural information about the host carbohydrate can be obtained without needing to solve the experimental problems that are associated with making CD measurements in the far-UV.

In the present work, results are reported from a CD spectroscopic study of the interaction of various dyes that are either active or inactive in modifying the structure of *A. xylinum* cellulose with a series of cello-oligosaccharides and with methylcellulose, hydroxypropylcellulose (HPC), linear malto-oligosaccharides, cyclomalto-oligosaccharides (cyclodextrins), and soluble starch (amylose). Cyclodextrins are included because of the general interest they have inspired as stationary phases in chiral chromatographic applications. The dyes that are capable of binding to the higher cello-oligomers are the same active dyes, **9**–**15**, that modify the structure of bacterial cellulose (Table 1). Induced CD spectra were not observed for any of the inactive dyes **16**–**22** in Table 1. Equilibrium constants for several dye–oligosaccharide association reactions are reported, and the possible structural features of the molecular complexes are discussed. Also included is a suggestion for an interesting analogy between the binding of water-soluble dyes to cello-oligosaccharides and the binding of the cellulose-degrading enzyme, cellobiohydrolase I, to cellulose.

## 2. Experimental

*Materials.*—Preparations and characterization of the fully acetylated derivatives of the cello-oligosaccharides **3**–**8** have been described before [30]. The free sugars **3**–**8** were obtained by transesterification with a catalytic amount of sodium methoxide in methanol at ambient temperature. The oligosaccharides generally crystallized from the reaction mixture and had physical properties in full accord with previously reported values [31].

Cellobiose, methylcellulose, HPC, the malto-oligosaccharides (Sigma Chemical Co.), and the cyclomaltoheptaose and cyclomaltooctaose ($\beta$- and $\gamma$-cyclodextrins, American Maize Co.) were commercial materials and were used as received. Congo Red **10**, was twice recrystallized before use. All other dyes were commercial materials of certified purity.

*Methods.*—CD spectra were measured using a Jasco 500A automatic recording spectropolarimeter (Japan Scientific Co. Inc., Easton, MD) with an IF-500 accessory interfacing the instrument to a personal computer for data collection and manipulation. Wavelength range, sensitivity scale, scan rate, and repeat function parameters were selected that gave the optimum signal-to-noise ratios for each of the dyes. A solution pathlength of 1 cm was used for all measurements. Sensitivity scales were calibrated daily using a 0.025% w/w solution of androsterone in dioxane.

Reagent stock solutions were prepared by dissolving the sugars and dyes separately in pH 7 phosphate buffer (0.2 M ionic strength). Volume aliquots taken from the sugars and each of the dye stock solutions were mixed and diluted to volume with buffer in order to prepare the final solution concentrations. CD spectra were measured over a wavelength range that was wider than the dye absorption bands in order to establish the baseline. Corrections for the cell blank and instrument baseline were made by subtracting the spectrum for the appropriately diluted buffered sugar solution by itself.

CD data for the determination of formation constants for the dye–sugar complexes were measured for a series of solutions in which the dye concentration was kept constant at 0.05 mM and the sugar concentrations were systematically increased. A low dye concentration is necessary to avoid excessive signal noise and to stay within the dynamic range of the CD instrument. Saccharide concentrations were always in excess of 0.05 mM so that the dye was the limiting reagent. The relatively low dye concentrations and the competing association equilibrium reactions were expected to minimize the extent of dye stacking interactions. Spectral measurements were made for as many as six solutions over the concentration range of 0.1–10.0 mM for the saccharides. Concentrations in the upper range were used when molecular association reactions were weak. Amylose and the hepta- and octa-mers of cellulose required heating to dissolve in the buffer solutions. Methylcellulose never completely dissolved even after extended periods of heating.

## 3. Results and discussion

The dyes **9–15** listed in Table 1 are known to prevent the assembly of crystalline cellulose by *A. xylinum* [12]. Solutions were prepared for each of these dyes in the presence of methylcellulose, HPC, and the series of oligosaccharides from cellobiose, **2** through cellooctaose, **8**. An induced CD signal was observed for all the dyes with all the sugars with the exception of **2** and **3**. For Calcofluor White, the first dye reported to affect the normal growth of bacterial cellulose, the induced CD signals from its association with the series of cello-oliogomers are extremely small and of such poor quality with respect to noise that formation constants could not be calculated. Spectra for the direct dyes **10–15** with cellohexaose, on the other hand, are much more intense and with absorbances reaching further into the visible region; hence, the signal quality is better (Fig. 1).

A common pattern is observed for all of the induced CD spectra in which the longest wavelength Cotton effect is negative in sign and more intense than the immediately adjacent positive band that maximizes at a shorter wavelength. Under equimolar dye conditions, the intensity of the induced spectrum is greatest for Trypan Blue. For this dye the negative and positive band maxima occur at much longer wavelengths, and the appearance of a third band of major proportions can be seen that has a negative maximum around 440 nm. This shorter wavelength negative band is actually induced for all of the direct dyes, but only for Trypan Blue and direct violet is it observed in the visible range. The spectral pattern of Fig. 1 is repeated for the association reactions of a given dye, e.g., Congo Red, with the individuals of the cellulose series (Fig. 2).

The spectra are equivalent in appearance for the cello-oligomer series and methylcellulose, differing only in their relative intensities. Cellotetraose was the shortest member of

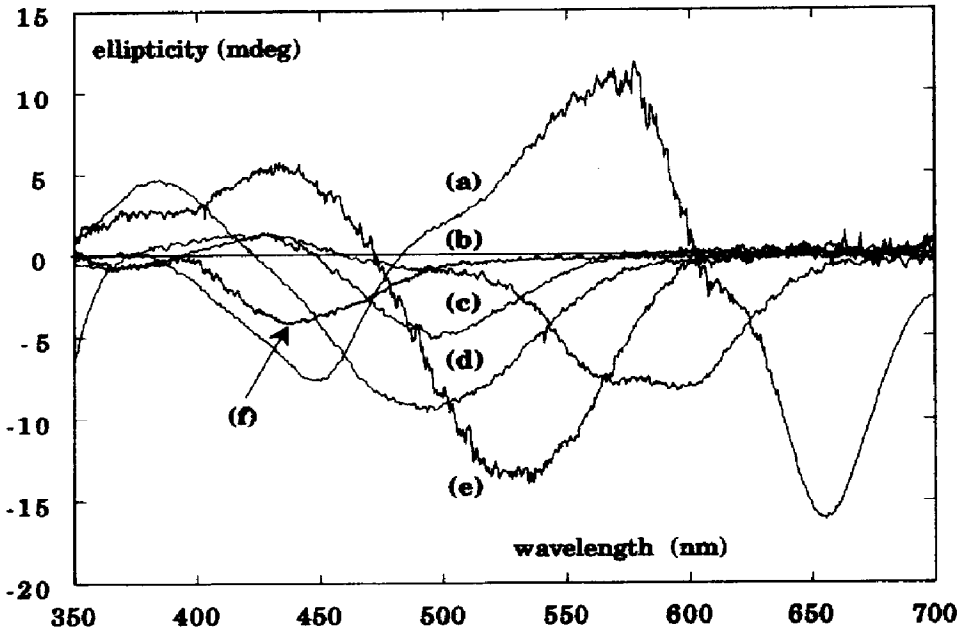



Fig. 1. Induced CD spectra for complexes formed between cellohexaose and the Direct dyes: (a) Trypan Blue; (b) Direct Violet No. 1; (c) Direct Yellow No. 1; (d) Direct Orange No. 8; (e) Congo Red; and (f) Titan Yellow at equimolar concentrations in aqueous pH 7 phosphate buffer.

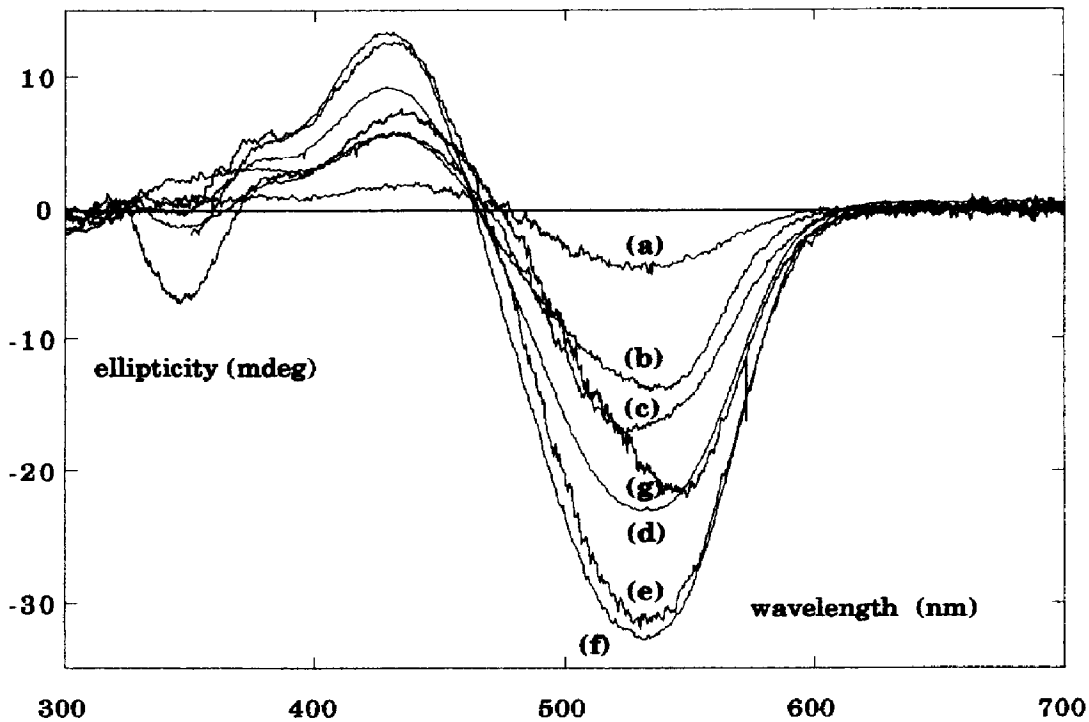



Fig. 2. Induced CD spectra for complexes formed between Congo Red and: (a) cellotetraose; (b) methylcellulose; (c) cellopentaose; (d) cellooctaose; (e) cellohexaose; (f) celloheptaose; and (g) hydroxypropylcellulose in pH 7 phosphate buffer. The oligomers are at equimolar concentrations. Concentrations of the polymers are not exactly defined because of uncertainty in the molar masses and the relative insolubility of (b).

Table 2
Formation constants and molar ellipticities for direct dye complexes with a series of cellulose oligomers and hydroxypropylcellulose (HPC)

| | -tetraose | -pentaose | -hexaose | -heptaose | -octaose | HPC [a] |
|---|---|---|---|---|---|---|
| Congo Red | | | (530 nm) | | | |
| $K_{DS}$ | 40 | 100 | 410 | 995 | 1000 | 1800 |
| $\theta_{DS}$ | −780 | −1700 | −1230 | −940 | −800 | −400 |
| Direct Orange No. 8 | | | (485 nm) | | | |
| $K_{DS}$ | | 120 | 400 | 830 | | |
| $\theta_{DS}$ | | −280 | −280 | −220 | | |
| Direct Yellow No. 1 | | | (490 nm) | | | |
| $K_{DS}$ | | | 700 | | | |
| $\theta_{DS}$ | | | −150 | | | |
| Direct Violet No. 1 | | | (595 nm) | | | |
| $K_{DS}$ | | 190 | 140 | 220 | | |
| $\theta_{DS}$ | | −80 | −350 | −290 | | |
| Trypan Blue | | | (660 nm) | | | |
| $K_{DS}$ | | | 2580 | | | |
| $\theta_{DS}$ | | | −510 | | | |
| Titan Yellow | | | (436 nm) | | | |
| $K_{DS}$ | | | 498 | | | |
| $\theta_{DS}$ | | | −152 | | | |

[a] Formula weight normalized to the heptamer (1765 g/mol).

the homologous series for which there was substantial evidence for CD induction into the absorption bands of Congo Red. A red shift of approximately 15 nm for the longest wavelength negative maximum is observed for the complex with HPC (Fig. 2g). Since the spectrum for methylcellulose conforms with the spectra for the oligomers, the shift for HPC is probably caused by involvement of either the bulkier side chain and/or the –OH functional groups in the association mechanism.

Induced CD spectra were not observed for the dyes of other structural types in the second column of Table 2. Dyes **16** and **17** were among those studied by Haigler and co-workers [10] and were reported not to alter the nature of *A. xylinum* cellulose to any significant degree [12]. It appears, therefore, that the dyes that are capable of binding to cellulose in the solid state and of altering the morphology of bacterial cellulose, are also capable of binding to cellulose oligomers, methylcellulose, and HPC in aqueous solution.

Calcofluor and the Direct dyes also bind to amylose, to $\beta$- and $\gamma$-cyclodextrins, and to higher members in the malto-oligomer series (Figs. 3 and 4). The shortest malto-oligomer for which there was substantial positive evidence for CD induction was the hexamer. That the degree of polymerization is longer is explained by the fact that overall the induced CD signals for the malto-oligomer series are much less intense than those for the equivalent members of the cello-oligomer series. Again a common CD spectral pattern exists which turns out to be the inverse of the pattern observed for the dye–cellulose complexes, i.e., the longest wavelength band is positive, etc. One peculiar exception to the regular pattern for

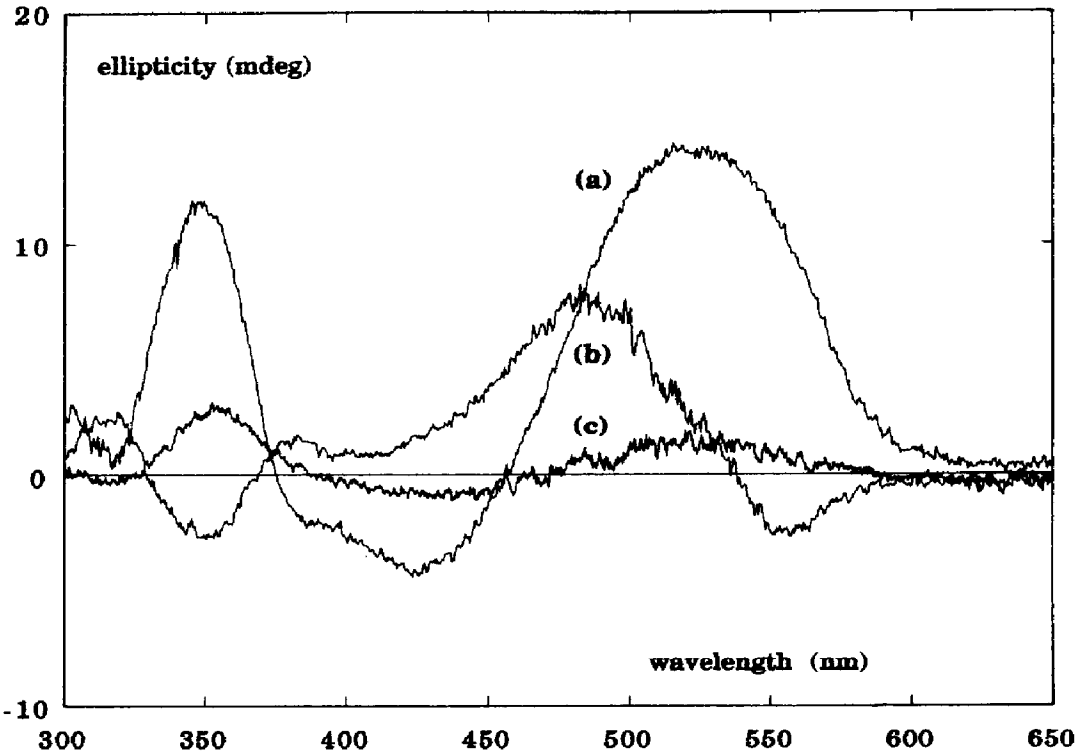



Fig. 3. Induced CD spectra for complexes formed between Congo Red and: (a) amylose; (b) maltoheptaose; and (c) maltohexaose in pH 7 phosphate buffer. The oligomers are at equimolar concentrations. The amylose concentration is not exactly defined because of the uncertainty in the molar mass.

complexes with Congo Red, however, is the spectrum for the maltoheptaose complex which shows a relatively large, as yet unexplained, blue shift and a new low intensity negative band that maximizes around 550 nm. The same spectrum was obtained using maltoheptaose samples from three independent sources and is not therefore explained by irregularities that might be present from the preparations.

Induced CD spectra in the visible range originate entirely with the molecular complexes because, when separate, the dyes absorb but are optically inactive, and the sugar molecules are optically active but do not absorb. Being a modification of absorbance detection, the correlation of measured signal size with concentration is properly described by Beer's law. So the observed intensity of the induced CD signal or ellipticity at any wavelength, $\Psi_{DS}$, is determined by two multiplicative factors, namely, the number of dye molecules that are complexed, determined by the formation constant $K_{DS}$ for the complexation reaction, and the induced rotational strength for the association complex, which is proportional to the molar induced ellipticity, $\theta_{DS}$. D, S, and DS refer to dye, sugar, and complex respectively.

The mathematical model of Benesi and Hildebrand [32] was used to calculate $K_{DS}$ for those systems where complexation occurred and where the hosts were available in sufficient quantities to make a series of measurements. In this model the concentration of one of the participants is kept constant while the other is changed. Spectra that are typical of a series are shown for Congo Red with cellohexaose in Fig. 5.

No significant changes in the spectra are observed as the carbohydrate concentration is increased. The assumption is made, therefore, that under the chosen conditions where the

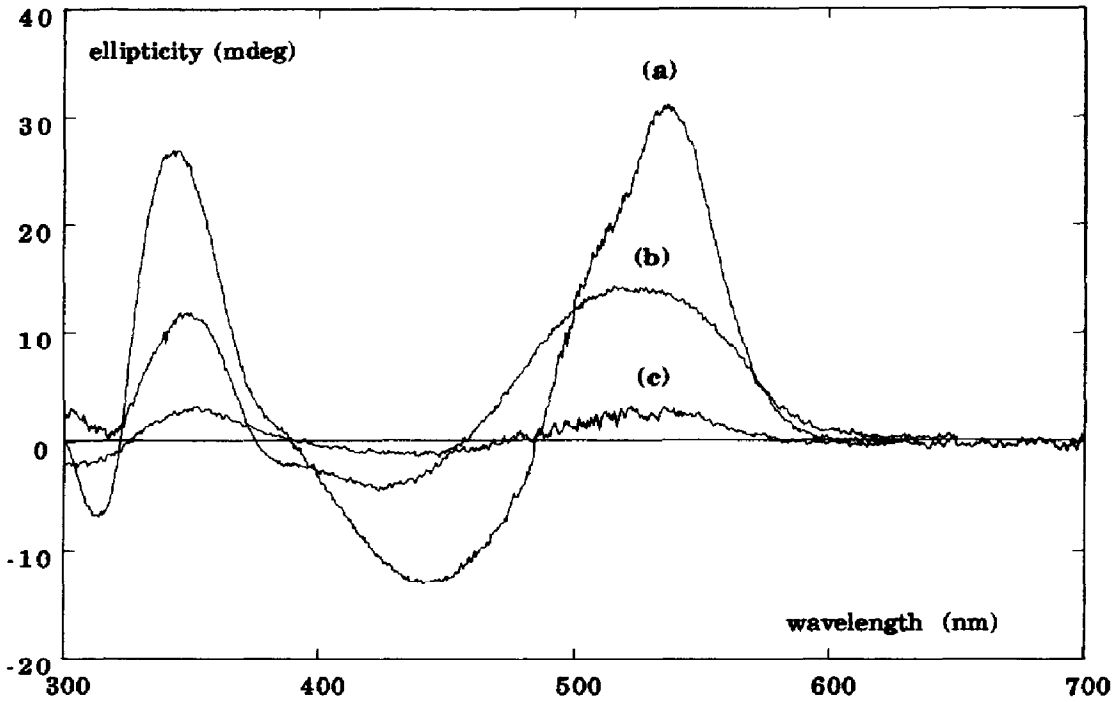



Fig. 4. Induced CD spectra for complexes formed between Congo Red and: (a) cyclo-malto-octaose ($\gamma$-cyclodextrin, 0.14 mM); (b) amylose; and (c) cyclo-maltoheptaose ($\beta$-cyclodextrin, 2.78 mM) in pH 7 phosphate buffer.

dye is the limiting reagent, the stoichiometry of the complex is 1:1. While this is a reasonable assumption and can be mathematically confirmed by the Benesi–Hildebrand model when the host is a molecule of low molar mass, the number of potential binding sites and the stoichiometry, would be expected to be $>1$ when the host is polymeric, e.g., methylcellulose, HPC, or amylose. In order to compensate for uncertainties in the molar masses of these polymeric forms, and to include the idea that a particular minimum repeat length is essential for complexation, formation constants for HPC and starch amylose were calculated using analytical concentrations that were normalized in terms of the molar mass of the hexamer or heptamer as a repeating unit.

Formation constants were calculated using the system of three equations for 1:1 stoichiometry:

$$(b[\mathrm{D}][\mathrm{S}]/\Psi_{\mathrm{DS}}) = \{([\mathrm{D}] + [\mathrm{S}] - [\mathrm{DS}])/\theta_{\mathrm{DS}}\} - (1/K_{\mathrm{DS}}\,\theta_{\mathrm{DS}}) \quad (1)$$

$$K_{\mathrm{DS}} = [\mathrm{DS}]/[\mathrm{D}][\mathrm{S}] \quad (2)$$

$$C_{\mathrm{D}} = [\mathrm{D}] + [\mathrm{DS}]; \quad C_{\mathrm{S}} = [\mathrm{S}] + [\mathrm{DS}] \quad (3)$$

where $b$ is the pathlength; $C_{\mathrm{D}}$ and $[\mathrm{D}]$, and $C_{\mathrm{S}}$ and $[\mathrm{S}]$ are the analytical and equilibrium concentrations for the dye and sugar respectively; and $[\mathrm{DS}]$ is the equilibrium concentration for the complex. The three-equation data set is repeated for measurements made for each incremental sugar concentration in the series with one dye. Because both $\theta_{\mathrm{DS}}$ and $K_{\mathrm{DS}}$ are unknowns, the simultaneous solution is done by iteration [32,33]. The procedure was initiated by assuming that $[\mathrm{DS}] = 0$ and terminated when successive values for $K_{\mathrm{DS}}$ con-

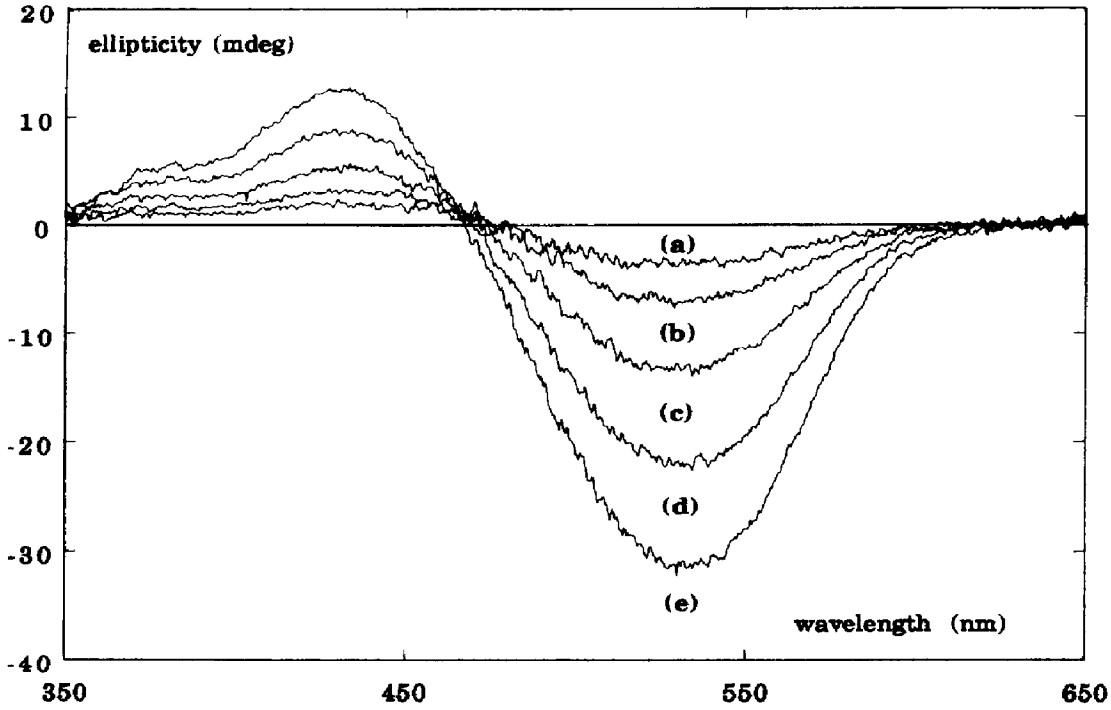



Fig. 5. Induced CD spectra for solutions of Congo Red and cellohexaose as a function of the cellohexaose concentration: (a) 0.17 mM; (b) 0.34 mM; (c) 1.37 mM; (d) 2.75 mM; and (e) 6.87 mM, in pH 7 phosphate buffer.

verged to within $\pm 10$. A failure to converge would be evidence for a wrong assumption regarding the stoichiometry of the complex.

Calculated values for $K_{DS}$ and $\theta_{DS}$ are given for the cellulose series in Table 2. Only experimental data measured at the wavelengths of the maxima for the most intense bands were used. While values for $K_{DS}$ are directly comparable within and among different dye systems, $\theta_{DS}$ values are wavelength dependent and can only be compared for a given dye with a series of hosts.

The most complete study was done for complexes that involved Congo Red. In the series of cello-oligomers, $K_{DS}$ increases with the molar mass of the hosts and reaches a maximum value at the heptamer. Formation constants could not be determined for methylcellulose–dye complexes because of the limited solubility of methylcellulose in aqueous buffers. The figure reported for the Congo Red–HPC complex (Table 2) was calculated using a stock solution concentration for HPC that was normalized to the molar mass of the heptamer. It differs from the $K_{DS}$ value for the celloheptamer by only a factor of two. Molar ellipticities, on the other hand, appear to maximize at the pentamer or hexamer. If the magnitude of this induced rotatory strength is indicative of the space-filling efficiency of the molecular fit, it might be concluded that the mutual molecular dimensions are the most complementary towards complexation when the saccharide is of that approximate size. The resultant $\theta_{DS}$ calculated for the HPC–Congo Red complex is independent of whether the HPC stock solution concentration is calculated using the molar mass for the hexamer or the heptamer.

By comparison, spectra for the maltooligomer–Congo Red systems are much less intense. In fact, only for maltoheptaose were the signals large enough to give us confidence in the

Table 3
Formation constants and molar ellipticities for Congo red and direct yellow complexes with a series of maltoses.

| | Maltoheptaose | Cyclomaltoheptaose | Cyclomalto-octaose | Amylose [a] |
|---|---|---|---|---|
| Congo Red | | | | |
| $K_{DS}$ | 26 | 36 | 44600 | 1970 |
| $\theta_{DS}$ | 250 | 700 | 760 | 420 |
| Direct Yellow No. 1 | | | | |
| $K_{DS}$ | | | 700 | |
| $\theta_{DS}$ | | | 7100 | |

[a] Formula weight normalized to the heptamer (1155 g/mol).

values that were calculated for $K_{DS}$ and $\theta_{DS}$ (Table 3). A weak complex of comparable stability is formed between Congo Red and the other maltoheptaose, namely β-cyclodextrin. The difference in molar ellipticities is real, however, and is probably a reflection of the effect of the rigidity of the closed structure over a more open, extended structure. The complex with γ-cyclodextrin is the most stable of all the maltose complexes. Interestingly $\theta_{DS}$ values for the two cyclodextrins are comparable in size, so the huge differences in the intensities of the induced CD signals and in the formation constants are thermodynamic in origin. The spectrum for the starch–amylose complex is very similar to those for maltohexaose and the cyclodextrins. It does show some loss of structure and a small blue shift in the longest wavelength band, and evidence of structure in the negative band. The positive Cotton effect maximizing at 340 nm is a firm result for all three structures. When the amylose concentration is expressed in terms of the molar mass of the heptamer, the formation constant is two orders of magnitude greater than that for maltoheptaose, but molar ellipticities are comparable in magnitude.

Calculations for the other Direct dyes are fewer in number because quantities for some of the sugars were quite limited. However from the partial lists for Direct Orange No. 8 and Direct Violet No. 1, the trends in the values for $K_{DS}$ and $\theta_{DS}$ that were seen for Congo Red appear to be repeated. Evidence for complexation with cellotetraose was observed, but spectra were too noisy to allow us to calculate $K_{DS}$ and $\theta_{DS}$. There are no obvious systematic structural differences from dye to dye that would explain the wide range in values for $K_{DS}$ and $\theta_{DS}$, for the cellohexaose complexes (Table 2). Several potential sites for hydrogen bonding exist, because all dyes have polar substituents, but only Trypan Blue has extra nonpolar substituents. Their presence might be the most significant factor in its complex with cellohexaose having the greatest stability, and perhaps pointing therefore to the relative importance of hydrophobic interactions that are commonplace in binding to the cyclodextrins. Another factor might be the degree of linearity. Direct Violet No. 1, for instance, has the least linearity and forms the least stable complex, while Direct Yellow No. 1 is the most linear and most stable.

With the single exception of Titan Yellow, the dyes that bind are structurally similar in that they all have three aromatic moieties disposed at ca. 1.05 nm separation distances along a rigid bis-diazo-pseudolinear structure that is centered around a biphenyl functional group. The single bond linking the biphenyl rings would allow for restricted rotation between the molecular "halves", and might be the singular most important distinguishing property

between active and inactive dyes that allows the Direct dyes to yield to the helical nature of the oligomers and to bind while other structures do not. The central N–N $\sigma$-bond in the structure of Titan Yellow will also allow some rotational freedom. A "central" sigma bond is absent from the structures of all the inactive dyes, **16–22**.

The linear separations between adjacent aromatic groups in active dyes closely match the molecular separation distances between cellobiose units in the cellulose structure and, to a first approximation, the size and helical extension of the cellohexaose structure might just be the optimum to bind fully with a dye such as **10**, causing the CD intensity for the complex to maximize at this degree of polymerization. Shorter cello-oligomers would have at least one terminal dye aromatic ring system unassociated, with a correspondingly lower stability for the complex, and longer analogues would show a decreased stability because of "superfluous" unassociated monomeric units of the host. Oligomers even longer than the currently available octamer, however, would be disposed towards binding a second dye molecule.

Consistent with this interpretation of course is the observation that the formation constants for complexes of the cello-oligomer series maximize around the heptamer, and the "normalized" value for HPC is similar. This last observation is in keeping with the idea that the origin of the extrinsic CD is the helical structure of the chiral host and that, after one complete turn of the helix, there is little additional stabilization of the dye–sugar association complex as additional dye molecules are bound. Furthermore there are no significant spectral changes with increasing molar mass so there is no evidence for intermolecular interactions between bound dye molecules: cf. the spectral similarities mentioned before for the starch and maltose series. While this interpretation of the induced CD activity does rely on a helical structure for the saccharide, it does not establish that it was helical prior to complexation. On the other hand there are insinuations in the literature [24] that the cyclodextrins might be viewed as helical structures of zero pitch, in which case the similar results for the celloheptaose and $\beta$-cyclodextrin complexes might be reason to believe that the acyclic form is helical in solution too, and that the effects of end groups are small.

The reversal of sign for the induced CD signals when the same dye is complexed with a maltose versus a cellulose derivative would indicate opposite handedness for the helical forms in solution [25]. If the origins of the bi-signate structures of the CD spectra are related to exciton coupling between adjacent aromatic chromophores in the same molecule of a given dye, the question of the handedness of the turn might be resolved. The interpretation uses the concept that the dye molecules will rotate around the central sigma bond of the biphenyl moiety under the influence of the helix. In current exciton coupling theories, a clockwise dihedral angle, and therefore a right-handed disposition between the principal long rotation axes of the aromatic chromophores, is indicated when the signs of the bands, reading from long to short wavelength, are positive to negative, and vice versa. Accepting this as being applicable to chromophores that are originally collinear, the conclusion is reached that the starch molecule has the clockwise conformation, coincident with the assignment described by Yalpani [25], and the helical rotation of the celluloses is counter-clockwise.

Although determination of the precise mechanism of the dye–sugar binding interactions described above must await further studies, there is good precedent to favor hydrophobic

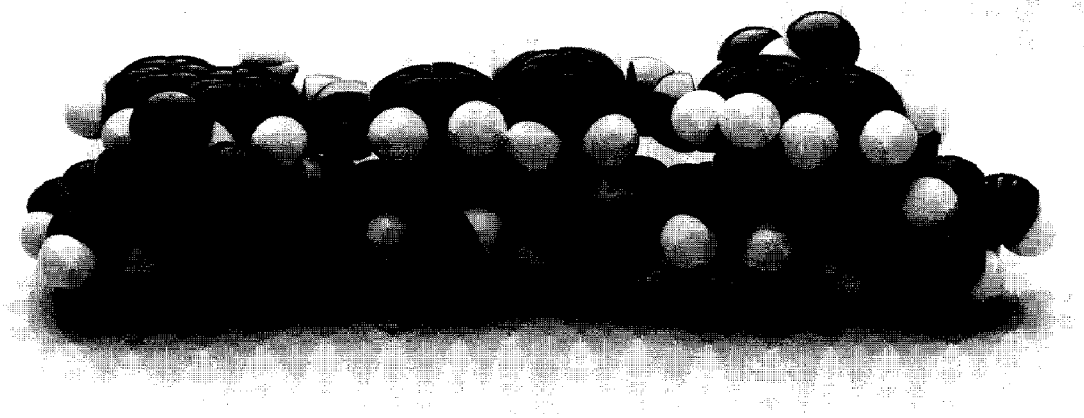


Fig. 6. Space-filling model of a probable structure for the complex between cellulose I and Congo red, showing its specific involvement with the hydrogens in the C-2, C-4, C-1′, C-3′, and C-5′ positions of the cellobiose repeat unit of the glycan chain.

association of aromatic species with sugars in aqueous media [34]. Nothing we have seen from the formation constant information in the current work would suggest an alternative mechanism. Induced CD spectra are observed for the dyes with cello-oligosaccharides in water but not in dimethyl sulfoxide. If specific hydrogen-bonding interactions linked the dyes to the sugars, one would expect to see association in nonaqueous solvents. Attempts to diminish the binding by the addition of the antihydrophobic agents urea and guanidinium chloride were nonquantitative, in part because the large amounts required would overcome the capacity of the buffer, and the accompanying pH change alters the degree of ionization, and therefore the binding, of the dye molecules. Furthermore, the dyes which do bind to the sugars vary greatly in the position and type of solubilizing groups present, making it unlikely again that hydrophilic interactions have to be specific in order to link the two species in aqueous solution.

In addition to the well-known cyclodextrin–benzenoid complexes [29], there is a growing awareness that many enzymes involved in sugar recognition and transport bear active sites in which aromatic rings stack on the sugar rings [35–38]. Thus the maltodextrin-binding protein of *E. coli* wedges maltose between four amino acid aromatic side chains [37]. It is reasonable to expect that the sugar–dye interaction takes the form of a hydrophobic association [34] between the aromatic rings of the dye and the C–H's of positions 2, 4, 1′, 3′, and 5′ of the cellobiose repeat unit in the glucan chain. Fig. 6 shows a space-filling model of cellohexaose (in the conformation derived from X-ray analysis of crystalline cellulose-I) with these hydrogens labelled [39], and a model of Congo Red superimposed upon these sites. An interaction of this type is indicated by several considerations.

We would also like to point out the possibility of a hydrophobic interaction in the binding of a cellulose-degrading enzyme to cellulose. The fungal cellulase, cellobiohydrolase I, from the wood-destroying organism *Trichoderma reesei* has been studied in detail [1,40,41] and shown to consist of a small (37 residue) cellulose-binding domain linked to a large glycoside-cleaving domain. The three-dimensional structure of the cellulose binding domain of this enzyme [42] possesses a coplanar array of three aromatic (tyrosine) residues with each of the three benzene ring centers separated by ca. 10.4 A, i.e., exactly the disposition

of the aromatic rings in dyes which bind to cello-oligosaccharides. Furthermore, the association constants reported for the binding of various cellobiohydrolases to cello-oligomers range from 730 to 62000 [43–46]; values that are reasonably consistent with the constants obtained here for the dye–oligomer complexes. It appears, therefore, quite possible that the mechanism of cellobiohydrolase I binding to its substrate cellulose and the mechanism of dye interaction with growing *A. xylinum* cellulose chains both involve the same type of hydrophobic interaction between sugar residues and aromatic rings.

## 4. Conclusions

The dyes that were reported to bind to and significantly alter the crystalline characteristics of bacterial cellulose pellicles are also capable of binding to cello-oligomers in a bulk aqueous medium. There is evidence from CD data for dye–sugar complexes to substantiate the idea that not only the polymeric saccharides but also the oligomers of cellulose and maltose have preferred helical conformations in aqueous solution. The opposite signs observed for the induced CD spectra between the maltose and cellulose series suggest that the helices turn in opposite angular directions; this is most likely an immediate consequence imposed by the different stereochemistries of the C-1–C-4′ linkages between monomeric units. Using the binding of dye molecules as a structural probe for carbohydrates in solution has brought us to the same conclusions that were reached from far-UV investigations of underivatized sugars. The visible range is experimentally much more convenient to use and should be fully exploited in future applications of other macromolecular interactions. The mechanism for the binding process is believed to be hydrophobic in nature, consistent with results from molecular association with cyclodextrin molecules and proteins.

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