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THE CIRCULAR DICHROIC SPECTRA OF SEVERAL SIALIC ACID-CONTAINING POLYSACCHARIDES ISOLATED FROM *Neisseria meningitidis**

HAROLD J. JENNINGS AND ROSS E. WILLIAMS
Division of Biological Sciences, National Research Council of Canada, Ottawa (Ontario), Canada, K1A 0R6

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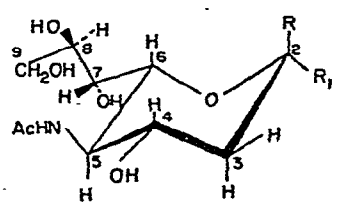
ABSTRACT

The circular dichroic spectra of the acid and sodium salt forms of several sialic acid-containing homo- and hetero-polysaccharides have been measured. The spectra are shown to be influenced by the state of ionization of the carboxyl groups contained in the sialic acid, the location within the individual sialic acid residues of the intersaccharide linkages, and changes in the configuration of hydroxyl groups remote to the carboxyl group of the sialic acid.

INTRODUCTION

Circular dichroism (c.d.) spectra of polysaccharides containing amide chromophores have been shown to be sensitive to the location of the intersaccharide linkages within the monomer residues, the configuration of the groups attached to carbon atoms involved in these linkages, and the overall conformation of the polysaccharide[1,2]. Because sialic acid (see diagram) contains an amide chromophore, the c.d. spectra of sialic acid-containing polysaccharides should also be sensitive to many of these same factors.



SIALIC ACID (*N*-ACETYLNEURAMINIC ACID)
α - anomer R = C^1O_2H, R^1 = OH
β - anomer R = OH, R^1 = C^1O_2H

*N.R.C.C. No. 15446.

Although the c.d. spectra of several monosaccharide sialic acids[3], sialic acid-containing oligosaccharides[4,5], and gangliosides[6] have been reported, only one previous study has examined the c.d. spectrum of a polysaccharide containing sialic acid residues namely, colominic acid)[4]. After having recently isolated a number of sialic acid-containing antigens from *Neisseria meningitidis* and characterized their structures by ^{13}C nuclear magnetic resonance[7,8], it seemed reasonable that a study of the c.d. spectra of these polysaccharides might also be useful in determining the sensitivity of the c.d. spectra of sialic acid-containing polysaccharides to the foregoing factors. In addition, the contribution of the sialic acid carboxyl chromophore to the c.d. spectrum of the polysaccharide might be ascertained and corroboration of the ^{13}C n.m.r. assignments of the α-configuration to the anomeric link in the sialic acid residues in all of the polysaccharides[7,8] might also be made.

RESULTS AND DISCUSSION

A. *Serogroup B and C polysaccharides.* — ^{13}C n.m.r. studies have indicated that the *N. meningitidis* serogroup B polysaccharide and colominic acid have identical structures, each consisting of α-(2→8)-linked sialic acid residues[7,8]. The c.d. spectrum of the free-acid form of the B polysaccharide is shown in Fig. 1A (compare Table I). Colominic acid (free acid), prepared from the sodium salt supplied by a commercial source, had a c.d. spectrum identical to that of the B polysaccharide. A c.d. spectrum of colominic acid has been reported previously[4]. At wavelengths where comparisons can be made, the previously reported spectrum was very similar to that reported here for the B polysaccharide.

The c.d. spectrum of the B polysaccharide (acid form) has two bands: one positive (196 nm) and one negative (233 nm) with crossovers at 216 and ~185 nm. That the negative band at 233 nm was associated with the presence of the carboxyl group in the polymer was gleaned from the fact that reduction of the carboxyl group eliminated the band entirely. The reduction also caused a slight shift (196→192 nm) and diminution in the intensity of the positive band [$\varepsilon_L - \varepsilon_R$ (192 nm) = +8.23 M^{-1} cm^{-1}]. The carboxyl group could be involved in the generation of the negative band either directly through the forbidden $n \rightarrow \pi^*$ carboxylic acid transition[5] or through perturbation by the carboxyl group of the $n \rightarrow \pi^*$ transition of the amide chromophore[9].

Upon formation of the sodium salt of the B polysaccharide, a shift to shorter wavelengths of the negative band occurred (233→230 nm) (Table I). This shift was accompanied by a slight shift to longer wavelengths of the positive band (196→198 nm) and a change in the position of the crossovers to 217 and 190 nm. The shifts in both bands were also accompanied by a significant decrease in intensity of the bands (230–235 nm band, 56% loss; 195–200 nm band, 25% loss). In this compound, the position and intensity of the negative band also was only slightly (<3%) influenced by variations in temperature (0→80°), salt concentration (0→1M NaF), and organic solvents (methanol, trifluoroethanol, 0→50%). This constancy would

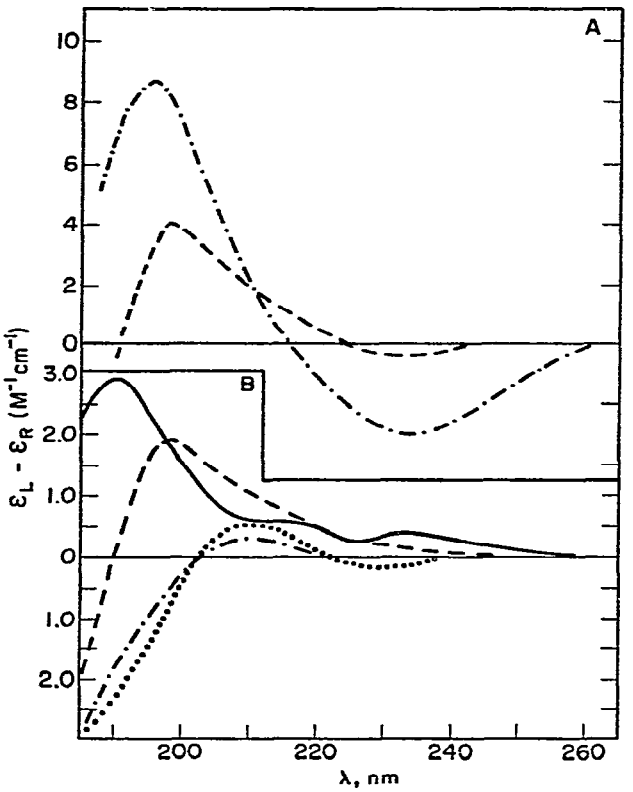


Fig. 1. The circular-dichroism spectra of sialic acid-containing mono- and poly-saccharides. A: (—·—·), B polysaccharide (free acid); (— — —), *O*-deacetylated C polysaccharide (free acid). B: (— — —), methyl α-ketoside (sodium salt); (———), methyl β-ketoside (sodium salt); (·····). W-135 polysaccharide (sodium salt); (—·—·), *O*-deacetylated C polysaccharide (sodium salt). Conditions: ~1 mg/ml, aqueous solution, 27°.

suggest that, if changes in long-range conformational order were occurring in the polymer, they did not influence the location and intensity of the negative band.

The intensity of the negative band was, however, influenced by the way in which the sialic acid monomers were linked. From ^{13}C n.m.r. experiments, the structure of the *O*-deacetylated *N. meningitidis* serogroup C polysaccharide has been shown to differ from that of the B polysaccharide only in the way in which the sialic acids are linked [α-(2→9) instead of α-(2→8)][7]. The c.d. spectrum of its free acid (Fig. 1A, Table I) was very different from that of the B polysaccharide. Although both polysaccharides had positive and negative bands in approximately the same location, the negative band in the *O*-deacetylated C polysaccharide was very much decreased in intensity and the crossover at 217 nm in the B polysaccharide shifted to 221 nm in the C polysaccharide. In addition, formation of the sodium salt of the *O*-deacetylated C polysaccharide led to complete loss of the negative band and no appreciable change in the location or intensity of the positive band at 198 nm (Table I).

TABLE I

CIRCULAR DICHROIC SPECTRA OF SIALIC ACID MONOMERS AND SEVERAL SIALIC ACID-CONTAINING POLYSACCHARIDES

Compounds	*C.d. bands*[a] [*Wavelength (nm)*, $\varepsilon_L - \varepsilon_R$ (M^{-1} cm^{-1})]	
	Free acid	*Sodium salt*
B-Polysaccharide	233, (−2.94) 216, crossover 210 sh, (+1.94) 196, (+8.54) ~185, crossover	230, (−1.30) 217, crossover 210 sh, (+2.08) 198, (+6.39) 190, crossover
C-Polysaccharide (*O*-deacetylated)	233, (−0.36) 221, crossover 210 sh, (+1.94) 198, (+3.94)	230–235, (——) 210 sh, (+2.08) 198, (+3.89) 192, crossover
Methyl α-ketoside of sialic acid[b]		235, (+0.18) 217 sh, (+0.79) 198, (+1.85) 191, crossover
Methyl β-ketoside of sialic acid[c]		235, (+0.36) 215 sh, (+0.65) 190, (+2.74) <185, crossover
N-Acetylneuraminic acid		230–235, (——) 210 sh, (+2.26) 193, (+4.11) <185, crossover
W-135 Polysaccharide	232, (−0.37) 224, crossover 210, (+0.98) 202, crossover	229, (−0.11) 217, crossover 211, (+0.53) 202, crossover
Y-Poly saccharide (*O*-deacetylated)	230, (−0.20) 224, crossover 210, (+0.67) 202, crossover	230–235, (——) 210, (+0.33) 202, crossover (?) 187, (−2.63)

[a]All spectra were taken in unbuffered aqueous solution (~1 mg compound/ml), 27°. Calculations are based upon weight of sample and mean residue molecular weight (see experimental details), sh = shoulder. [b]Methyl α-*N*-acetylneuraminic acid. [c]Methyl β-*N*-acetylneuraminic acid.

Comparison of the 230–240 nm region of the c.d. spectra of the sodium salts of the B and *O*-deacetylated C polysaccharides with the spectra of the sodium salts of two model compounds, the methyl α- and β-ketosides of sialic acid, was made (Fig. 1A, 1B, Table I). Ellipticity was observed in this region in the model compounds. However, its intensity and sign bore little resemblance to that observed in the

spectra of the polymers. The spectra of both model compounds displayed weak positive ellipticity in this region, in accord with the results of Kielich *et al.*[3]. These authors also noted that the magnitude and sign of the elllipticity in this region was dependent upon the aglycon substituent. Changing the aglycon substituent from methyl to ethyl or allyl resulted in a shift in the magnitude of the ellipticity to more-negative values (−0.035 and −0.10, respectively). This observation would suggest that groups somewhat removed from the anomeric carbon atom are able to influence the observed ellipticity in this region, presumably through either steric or electronic means (see later).

The difference in the magnitude of the ellipticity in this region between the methyl α- and β-ketosides of sialic acid (α, +0.18; β, +0.36) would suggest that this region of the c.d. spectrum might be used to assign the configuration of groups about the anomeric carbon atom. However, the dependency of the ellipticity in this region on groups far removed from the anomeric center would require that the assignment be made by comparing the c.d. spectra of the polymers with the c.d. spectra of models having all of the structural elements found in the polymers and differing only in the configuration about the anomeric center. Such models are not, at present, available.

Examination of the region in the c.d. spectrum near 200 nm showed that the location of the positive band in the 185–200 nm region of the c.d. spectrum of both the B and *O*-deacetylated C polysaccharides (as sodium salts, Table I) was unaffected by the change in the way the sialic acid monomers were linked to form the polymers. Only the magnitude of the ellipticity was diminished. The decrease in magnitude could arise from a change in the conformation of the polysaccharide or from a change in the magnitude of the electronic coupling associated with the chromophores.

Comparison of the 185–200 nm region in the c.d. spectra of the sodium salts of the two model compounds (the methyl α- and β-ketosides of sialic acid) with same region in the polymers suggested that, if a somewhat simplistic analysis is used, assignment of the anomeric configuration might be made from the position of the positive band between 185 and 200 nm. The c.d. spectra of the two model compounds are similar to those obtained by Kielich *et al.*[3]. Our results, however, indicate that a large difference between the location of the positive band-maxima exists (α, 198 nm; β, 190 nm). As a check, the spectrum of a sample of *N*-acetylneuraminic acid (NANA, sodium salt), which had been shown by ^{13}C n.m.r. to be mainly the β-anomer, both as its free acid[7] and sodium salt[8], was recorded and was found to show a positive band-maximum at 193 nm when the conditions were employed the same as those used to measure the spectra of the two model compounds. This value is the same as that found by Kielich *et al.*[3]. Dickinson and Bush[5], on the other hand, found that the positive band-maximum in NANA occurred at 199 nm. This difference could result from a difference in the conditions used to measure the spectra (water *vs.* 1.0 mM Tris-HCl, pH 7.5). If it may be assumed that the β configuration results in a positive band-maximum near 190 nm and the α configuration results in a positive band-maximum near 200 nm, then the linkage in both polysaccharides would be assigned as α, in accord with the ^{13}C n.m.r. results.

Even though the results of this simplistic analysis of the c.d. spectra agree with the ^{13}C n.m.r. results, the assignment of anomeric configuration from the c.d. spectra is tenuous at best, as the models used may not, in fact, be adequate. The ones used take no account of any long-range conformational effects and long-range electronic effects that might influence the spectra. These types of long-range effects have been previously noted in the c.d. spectra of polypeptides and polynucleotides[9,10].

B. *Serogroup W-135 and Y polysaccharides.* — The W-135 and *O*-deacetylated Y polysaccharides differ from those of the B and *O*-deacetylated C polysaccharides in that they do not contain contiguous residues of a sialic acid monomers. Both have been shown to have a repeating disaccharide sequence; the former contains the repeating unit 4-*O*-α-D-galactopyranosyl-β-*N*-acetylneuraminic acid and the latter, the repeating unit 4-*O*-α-D-glucopyranosyl-β-*N*-acetylneuraminic acid. From the ^{13}C n.m.r. experiments[11], it has been suggested that the sialic acid in both disaccharides is α-linked to C-6 in the hexose residue to form the polymer. As these polysaccharides differ only in the configuration of the hydroxyl group on C-4 of the hexose residue, it is not surprising that the c.d. spectra of their sodium salts are very similar (Fig. 1B, Table I). However, one notable difference is present. The c.d. spectrum of the W-135 polysaccharide has a weak negative band at 229 nm, whereas no band at all in this region was observed in the c.d. spectrum of the *O*-deacetylated Y polysaccharide. Thus, it would appear that measurements made in the 230-nm region of the c.d. spectrum are sensitive to small changes in the hexose unit of the aglycon. This effect is comparable to that observed in the sialic acid model-compounds, where methyl, ethyl and allyl groups were used as aglycon substituents[3] (see foregoing discussion). On the basis of the effects noted with the model compounds and the remoteness of the amide to the configurational changes at the C-4 of the hexose residue, it would seem reasonable to attribute the effect noted to effects on the carboxyl group. The presence or absence of ellipticity in the 230-nm region of the spectrum might then be attributed to local changes in the proximity of a group interacting with carboxyl group. The local change in proximity of groups might, in turn, be the result of either a simple change in configuration at C-4 in the hexose residue, or a more-complex change in long-range conformation of the polymer initiated by the change in the configuration of C-4 in the hexose residue. A choice between these two explanations is not possible with the information at hand.

Changing the sodium salts to the free acid form of the polysaccharide led to an increase in ellipticity near 230 nm in the spectrum of the W-135 polysaccharide and the appearance of ellipticity in this region of the spectrum of the *O*-deacetylated Y polysaccharide. The spectral differences noted between the c.d. spectra of the sodium salts of the two polymers were maintained. Thus, even in the free-acid from of the polysaccharides, the 230-nm region of the c.d. spectrum is sensitive to small changes in the aglycon group.

In addition to these variations near 230 nm, the positive band at 210 nm increased in intensity in both of the free-acid forms by approximately the same amount (namely, 2-fold).

Direct comparison of the spectra of the sodium salts of the two monomer models, the methyl α- and β-ketosides, with the spectra of the sodium salts of the two polysaccharides, W-135 and *O*-deacetylated Y, further illustrates the problems associated with using models of small molecular weight as a basis for interpreting the c.d. spectra of poly(sialic acids). The only seemingly common feature in each of the spectra is the position of the positive shoulder at 215–217 nm in the monomer models and the presence of the positive band near 210 nm in the polymers.

When the c.d. spectra of the sodium salts of the W-135 and *O*-deacetylated Y polysaccharides are compared with the c.d. spectra of the sodium salts of the B and *O*-deacetylated C polysaccharides, major differences between the systems are observed: (*1*) the intensity of the ellipticity in the 230-nm region in the W-135 and *O*-deacetylated Y polysaccharide spectra is decreased; (*2*) a weak, positive band at 210 nm in the spectra of W-135 and *O*-deacetylated Y polysaccharide appears; (*3*) the intense positive ellipticity at 198 nm present in the spectra of the B and *O*-deacetylated C polysaccharides is lost and is replaced by substantially negative ellipticity in the spectra of the W-135 and *O*-deacetylated Y polysaccharides.

Changing the sodium salt forms of the polysaccharides into the free-acid forms generally led to increases in negative ellipticity in the 230-nm region of the spectra or the appearance of negative ellipticity where there was none before. In addition, the positive ellipticity noted at 198 nm in the B polysaccharide and the positive ellipticity noted at 210 nm in the W-135 and *O*-deacetylated Y polysaccharide increased.

Whether these major differences in the spectra can be attributed entirely to the differences in linkages, namely the linkage of sialic acid to the hexose residue now (2→6) and the (1→4) linkage of the hexose residue to sialic acid, is difficult to ascertain. For the W-135 polysaccharide, however, some pertinent information could be obtained from c.d. studies of several model trisaccharides from milk[5]. One of the trisaccharides [(2→6)-α-*N*-acetylneuraminyllactose] contains a disaccharide situated at the non-reducing end in which the *N*-acetylneuraminic acid residue is α-(2→6)-linked to a galactose group. This linkage is also found in the W-135 polysaccharide. The c.d. spectrum of the trisaccharide had a negative band at 226 nm ($\varepsilon_L - \varepsilon_R = +0.15$) and a positive one at 199 nm ($\varepsilon_L - \varepsilon_R = +1.15$), with a shoulder at 211 nm ($\varepsilon_L - \varepsilon_R = +0.24$). The position and intensity of the negative band at 226 nm and the positive shoulder at 211 nm are almost identical to the bands found in the sodium salt of the W-135 polysaccharide. The positive band at 199 nm in the trisaccharide had no equivalent in the spectrum of the polysaccharide, where only negative ellipticity was observed. Although it is tempting to speculate that the similarities between the spectrum of the milk trisaccharide and that of the W-135 polysaccharide are the result of the α-(2→6) link of the sialic acid to galactose, and the differences result from the position of the link of the hexose residue to the sialic acid (on C-4) and its proximity to the C-5 amide chromophore, caution must be exercised as the effects of these types of linkages are unknown and adequate models to assess this factor are not available.

As may be seen from the results the c.d. spectra of sialic acid-containing poly-

saccharides are complex and affected by the type of linkages that are involved in the formation of the polysaccharide. Therefore, information concerning the configurational type and position of the intersaccharide linkages in an unknown polysaccharide can be gained from c.d. spectra only if adequate models of the various types of linkages and their positional isomers have been prepared and some estimate of the long-range conformational and electronic effects have been made.

EXPERIMENTAL

Procedures. — The *N. meningitidis* serogroup B, C, Y, and W-135 (strains 608, 2241, 548, and 546, respectively) polysaccharide antigens were isolated and purified as previously described[7,10]. The *O*-acetylated native serogroup C and Y polysaccharides were *O*-deacetylated by incubation for 4 hours at 37° in 0.1M sodium hydroxide[7]. The free-acid form of the polysaccharides was produced by passage of a solution of the purified polysaccharides (isolated as their sodium salts) through Rexyn-101 (H^+) ion-exchange resin and subsequent lyophilization of the deionized solution. Colominic acid (*Escherichia coli*) was obtained from the Koch–Light Laboratories, Colnbrook, England. Reduced colominic acid was prepared essentially by the method of Onodera *et al.*[13]. The methyl ester used in this procedure was prepared by using a methanol-soluble fraction of colominic acid and treating it with diazomethane in methanol. *N*-Acetylneuraminic acid (sialic acid) was obtained from Nutritional Biochemicals Ltd., Cleveland, Ohio. The methyl α- and β-ketosides of sialic acid (namely, methyl α- and β-*N*-acetylneuraminic acid) were prepared according to the methods of Yu and Ledeen[14] and Kuhn *et al.*[15], respectively. The sodium salts of the acidic methyl glycosides were prepared by titration of a solution of the free acid to pH 7 by using 0.01M sodium hydroxide and subsequent lyophilization of the solution. Circular dichroism spectra were taken on a calibrated Cary–Varian spectropolarimeter[16] model 61. The quartz cells used, 0.05-cm path, were thermostated at 27 ±0.5°. Solutions (approximately 1 mg/ml) were made up in deionized, glass-distilled water unless otherwise stated. The molar extinction coefficients $\varepsilon_L - \varepsilon_R$ ($M^{-1}\,cm^{-1}$) were calculated in the usual manner[17]. Mean-residue concentrations were used for the purposes of the calculations. They were calculated from the dry weight of the sample and the following mean-residue molecular weights: *N*-acetylneuraminic acid, 309; methyl α- and β-*N*-acetylneuraminic acid, 323; serogroup B and C (*O*-deacetylated) polysaccharides, 291; and serogroup Y (*O*-deacetylated) and W-135 polysaccharides, 227. Molecular weights of the sodium salts of the foregoing acids were calculated by adding 22 to the values given.

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