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# Optical Activity and Conformation of Carbohydrates. II. Optical Rotatory Dispersion and Circular Dichroism Studies on Immunochemically Reactive Oligo- and Polysaccharides Containing Amino Sugars and Their Derivatives*

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ABSTRACT: Optical rotatory dispersion and circular dichroism spectra of several mono- and oligosaccharides are presented, as well as spectra of samples of teichoic acid, *N*-acetylneuraminic acid, *N*-acetylneuraminyllactose, and colominic acid, a polymer comprising repeating units of *N*-acetylneuraminic acid. Attention is focused on the optical activity generated by the 2-acetamido chromophore, common to all compounds examined. The circular dichroism spectra confirm the reality and spectral location of the Cotton effects in those optical rotatory dispersion spectra which have been previously reported. On the basis of the size of the Cotton effect troughs of these compounds and of other oligosaccharides whose rotations are given in previously published reports, it is possible to arrange the oligosaccharides containing *N*-acetyl-D-glucosamine residues into three groups: (a) oligosaccharides in which the *N*-acetyl-D-glucosamine residue is $\beta$ linked but unsubstituted; these compounds show the smallest Cotton effect troughs near 220 m$\mu$ (smaller than $-2500$ (deg cm$^2$)/dmole); (b) oligosaccharides which have their *N*-acetyl-D-glucosamine residues substituted either on C-3 or C-4 by $\beta$-D-Gal or $\alpha$-L-Fuc-(1 $\rightarrow$ 2)-$\beta$-D-Gal; these compounds have intermediate values for $[m]_{\text{trough}} - [m]_{300\ m\mu}$ (between $-3000$ and $-9000$ (deg cm$^2$)/dmole); (c) oligosaccharides in which the *N*-acetyl-D-glucosamine residue is disubstituted; these compounds have the largest corrected trough rotations (greater than $-9000$ (deg cm$^2$)/dmole). The circular dichroism spectra agree with the optical rotatory dispersion data ordering except in one instance: lacto-*N*-fucopentaose I exhibits a much more intense band near 210 m$\mu$ than does lacto-*N*-fucopentaose II, whereas the reverse is true in the optical rotatory dispersion spectra. The discrepancy is resolved by casting the data in such a way as to take into account the strong influence of monofucosyl substitution on the *N*-acetyl-D-glucosamine Cotton effects and ellipticity bands. Tentative rules are proposed which may allow decisions to be made as to the nature and position of substituents on *N*-acetyl-D-glucosamine residues in oligo- and polysaccharides. It is shown that the *N*-acetyl-D-glucosamine residues in teichoic acids dominate the shape and largely determine the intensity of optical rotatory dispersion spectra of these polymers. While the optical rotatory dispersion and circular dichroism studies cannot distinguish mixtures of $\alpha$- and $\beta$-teichoic acids from both linkages on a single polyribitol phosphate backbone, which is readily demonstrated immunochemically, they do provide a rapid estimate of the proportion of $\alpha$ to $\beta$ in each case. The great differences in spectra of colominic acid and of its constituent *N*-acetylneuraminic acid suggest a conformational dependence of the optically active absorption bands in the polymer. This finding and the possible applicability of the work reported here to diverse immunochemical studies are discussed.

In the first paper of this series (Beychok and Kabat, 1965) the optical rotatory dispersion spectra of mono-, oligo-, and polysaccharides containing the 2-acetamido (*N*-acetyl) group were described. It was shown that this chromophore generates a characteristic Cotton effect centered between 210 and 215 m$\mu$. From the intensity and sign of the long-wavelength extremum of this

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Cotton effect (usually a trough) and from the dispersion curve at longer wavelengths, it was possible to infer analytical and structural features of amino sugars and complex biological substances containing amino sugar residues. Among preliminary findings of interest were (1) the optical rotatory dispersion spectra are valuable adjuncts in determining whether *N*-acetylated amino sugar residues are $\alpha$ or $\beta$ linked in oligosaccharides; (2) the extent and kind of substitutions on the *N*-acetylated amino sugar residues may, in certain cases, be determined or confirmed by rotatory dispersion studies; (3) application of empirical symmetry rules for optical activity leads to apparently correct inferences about the preferred orientation of the planar amide group with respect to the ring; (4) conformational features in polymeric substances, such as interactions among residues due to periodicity or exposure of the 2-acetamido group to solvent water may be revealed or are, at least in principle, capable of detection; (5) the Cotton effect trough near 220 m$\mu$ is often very intense, the residue rotation occasionally exceeding in magnitude that observed for the n–$\pi^*$ band of the peptide bond in the $\alpha$ helix; analysis of helix content in glycoproteins containing significant quantities of *N*-acetylglucosamine or its derivatives must therefore take into account these substantial rotations.

Subsequent work in our laboratories (Lloyd *et al.*, 1967, 1968) and in those of others (Arcos and Lieberman, 1967) has confirmed the anticipated analytical utility of these measurements, especially when only milligram quantities of material are available. For example, the determination of structures of difucosyl and other oligosaccharides produced by alkaline degradation of blood group A, B, H, and $Le^a$ substances was materially assisted by analysis of optical rotatory dispersion and circular dichroism spectra (Lloyd *et al.*, 1967, 1968).

Any conclusions about the conformation and environment of the 2-acetamido chromophore rest upon the ability to separate the rotatory contribution of this group from those of other optically active centers or groups in the sugar. In optical rotatory dispersion spectra, this is necessarily difficult to accomplish and approximations are usually crude. Beychok and Kabat (1965) simply subtracted the rotation at 300 m$\mu$ from that at the trough wavelength to take account of very different background rotations in widely different acetamido-containing compounds. Obviously, a more meaningful isolation of the contributions of a particular chromophore requires measurements of circular dichroism spectra, which are presented in this paper for many of the compounds whose optical rotatory dispersion spectra were previously reported. It should be emphasized, however, that background rotations, when present, may assist in empirical, diagnostic studies intended to distinguish different compounds or structures even when the theoretical significance of observed differences is not at all understood. Thus, for compounds not previously reported at all, we present here both optical rotatory dispersion and circular dichroism spectra whenever feasible, the former because differentiation among closely related substances may be greatly facilitated with the aid of such spectra, and the latter because any semiempirical or theoretical methods for conformation analysis strictly require the assignments of particular bands (Cotton effects or ellipticity bands) to particular chromophoric transitions.

In the present work, attention is focused on the optical activity of the 2-acetamido chromophore. In addition to several mono- and oligosaccharides, data are presented for samples of teichoic acid, 5-acetamido-3,5-dideoxy-D-glycero-$\alpha$-D-mannononulopyranosonic acid, often called *N*-acetylneuraminic acid, 5-acetamido-3,5-dideoxy-D-glycero-$\alpha$-D-mannononulopyranosonic acid-(2→3)-$\beta$-D-galactopyranosyl-(1→4)-D-glucose, conventionally called *N*-acetylneuraminyllactose, from bovine colostrum and colominic acid, a polymer made up of repeating units of *N*-acetylneuraminic acid.

## Materials

The preparations and sources of *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, their glycosides, the galactosyl-*N*-acetyl-D-glucosamines, and the milk oligosaccharides are those given in the earlier paper (Beychok and Kabat, 1965). *N*-Acetylneuraminic acid and *N*-acetylneuraminyllactose were obtained from Calbiochem Corp and a sample of *N*-acetylneuraminic acid from meconium was also supplied by Dr. Karl Meyer. The following oligosaccharides were also studied: $\beta$-D-GNAc-(1→3)-D-Gal,[1] $\beta$-D-GNAc-(1→6)-D-Gal, and $\beta$-D-GNAc-(1→3)-[$\beta$-D-GNAc-(1→6)]-D-Gal from Dr. Z. Yosizawa, Tohoku University (Yosizawa, 1961); $\beta$-D-Gal-(1→3)-D-GalNAc from Dr. H. Flowers of the Weizmann Institute (Flowers and Shapiro, 1965); $\beta$-L-Fuc-(1→3)-D-GNAc from Dr. R. Jeanloz (Rachaman and Jeanloz, 1968). This was reported to be the $\alpha$ compound but Dr. Jeanloz has asked us to state that it is actually the $\beta$ compound. This is consistent with findings of Dr. Sigfrid Svensson in this laboratory indicating that it is poorer than $\alpha$-fucosyl derivatives or L-fucose in inhibiting precipitation of eel anti-H by a fucogalactan from *Polyporus borealis* (S. Svensson and E. A. Kabat, unpublished data).

Four samples of teichoic acids from *Staphylococcus aureus* were studied: (1) a sample from the *Copenhagen* strain contained 15% $\alpha$- and 85% $\beta$-linked GNAc from Dr. J. L. Strominger (Sanderson *et al.*, 1961, 1962) which was shown to be a mixture of $\alpha$- and $\beta$-linked polymers (Torii *et al.*, 1964; Nathenson *et al.*, 1966; *cf.* Kabat, 1968); (2) one from the *NYH*-6 strain from Morse (1962) was considered to contain approximately 50% each of $\alpha$- and $\beta$-linked GNAc (Torii *et al.*, 1964); (3) a sample from strain *A*1 from Dr. J. Baddiley, Newcastle (Davidson *et al.*, 1964), containing only $\beta$-linked GNAc; and (4) a sample from strain *3528* from Drs. J. L. Strominger and D. J. Tipper (Nathenson *et al.*, 1966) which contained 95–100% $\alpha$-linked GNAc.

[1] GNAc = *N*-acetyl-D-glucosamine; GalNAc = *N*-acetyl-D-galactosamine.

A sample of colominic acid, No. B7-Fr2, a polymer of *N*-acetylneuraminic acid containing over 98% *N*-acetylneuraminic acid, was kindly provided by Dr. G. T. Barry (Barry, 1958).

## Methods

Optical rotatory dispersion spectra were measured on a Bendix-Ericcsen Model 60 spectropolarimeter and, when it became available, a Cary 60 spectropolarimeter. About two-thirds of the time, the two instruments gave excellent agreement on first measurements. When agreement was not good, samples were rerun until satisfactory agreement was achieved. Almost always, one or the other instrument gave the correct result initially and the second instrument yielded a matching result after one or more reruns. It became apparent that the difficulty resided in wandering base lines, the underlying cause of which may be the high-intensity lamp of the Cary and is of uncertain origin in the Bendix-Ericcsen. No optical rotatory dispersion result is reported here which was not measured at least twice and sometimes many more times, each sample run having been bracketed by solvent base lines until they matched. This procedure seems to be absolutely required for adequate precision, at least at the dilutions which were usually employed in these measurements.

Circular dichroism spectra were measured on a modified Jouan dichrograph (Beychok, 1967) and, for the Lewis substance and oligosaccharides, a Cary Model 60 spectropolarimeter with circular dichroism attachment. When tested, no serious discrepancies between the two have thus far been detected. The Cary has a better signal:noise ratio above optical densities of about 0.8–1.0. The Jouan does not suffer from base-line instability and, in fact, gives the same base line for air as for an optically inactive solvent. Cell positioning, also, is not critical in the Jouan. For the oligosaccharides, comparisons between the two circular dichoism instruments have not been as extensive as between the two optical rotatory dispersion instruments. Steroids and polypeptides have, however, given excellent agreement in circular dichroism spectra.

Molecular ellipticity values, in (deg $cm^2$)/dmole, are employed in plotting the circular dichroism spectra.

All measurements were performed at room temperature.

## Results

Figure 1A,B shows the circular dichroism spectra of D-GNAc, Me-α-D-GNAc, Me-β-D-GNAc, D-GalNAc, Me-α-D-GalNAc, and Et-β-D-GalNAc in the wavelength interval 250–209 mμ. All of the circular dichroism bands, which are probably n–π* transitions of the amide group in all cases, are negative in sign. The circular dichroism spectra confirm the reality and spectral location of the Cotton effect in the previously reported optical rotatory dispersion spectra. In all but the case of Me-α-D-GalNAc, the difference

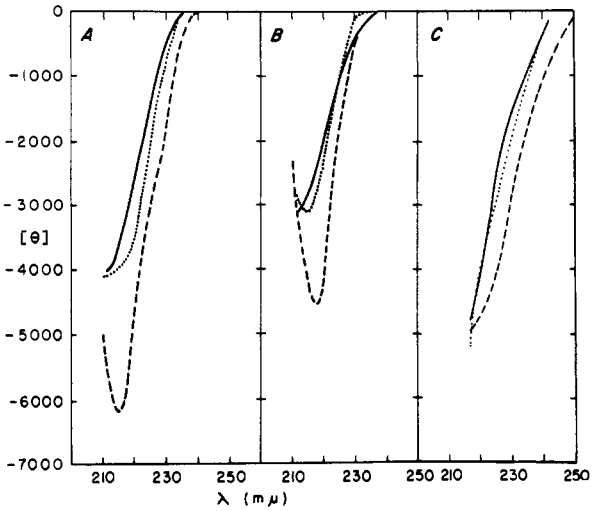


FIGURE 1: Circular dichroism spectra of mono- and disaccharides containing GNAc and GalNAc residues. (A) (—) D-GNAc; (·····) Me-α-D-GNAc; (---) Me-β-GNAc. (B) (—) D-GalNAc; (·····) Me-α-D-GalNAc; (---) Et-β-D-GalNAc. (C) (—) β-D-Gal-(1→6)-D-GNAc; (·····) β-D-Gal-(1→3)-D-GNAc; (---) β-D-Gal-(1→4)-D-GNAc.

calculation used earlier to decide the sign of the Cotton effect appears to have been valid and the attempts at gauging the relative magnitudes of the isolated Cotton effects were semiquantitatively successful. Thus, the optical rotatory dispersion results indicated that the acetamido group generated bands of relative strength Me-β-D-GNAc > Me-α-D-GNAc ≥ Et-β-D-GalNAc > Me-α-D-GalNAc. The circular dichroism band is most intense for Me-β-D-GNAc, but Et-β-D-GalNAc shows a slightly deeper band than Me-α-D-GNAc and Me-α-D-GalNAc is the weakest of the four. The optical rotatory dispersion data, furthermore, tend to amplify the difference between Me-β-D-GNAc and Me-α-D-GNAc. With Me-α-D-GalNAc, the optical rotatory dispersion curve appeared quite featureless and almost identical with that of methyl α-D-galactoside. In fact, the dispersion was less steep in an increasing positive direction for the galactoside than for the acetamido derivative. Thus, for purposes of distinguishing between α and β anomers of GNAc in various compounds and between α-linked *N*-acetylglucosamine and *N*-acetylgalactosamine residues, optical rotatory dispersion spectra are considerably more decisive than circular dichroism spectra, at least over the intervals studied.

The circular dichroism spectra of Figure 1 illustrate a difficulty which arises in these six compounds as well as in several other oligosaccharides shown below. In most of the early circular dichroism measurements, as well as in some of the more recent ones, instrumental limitations prevented measurements at wavelengths shorter than about 209–211 mμ. Even in the monosaccharides shown, there is a substantial range in wavelength of maximum ellipticity. Thus, in Me-β-D-GNAc, the maximum is observed at 215 mμ; in Et-β-D-GalNAc it occurs at 217–218 mμ; in GNAc and in GalNAc

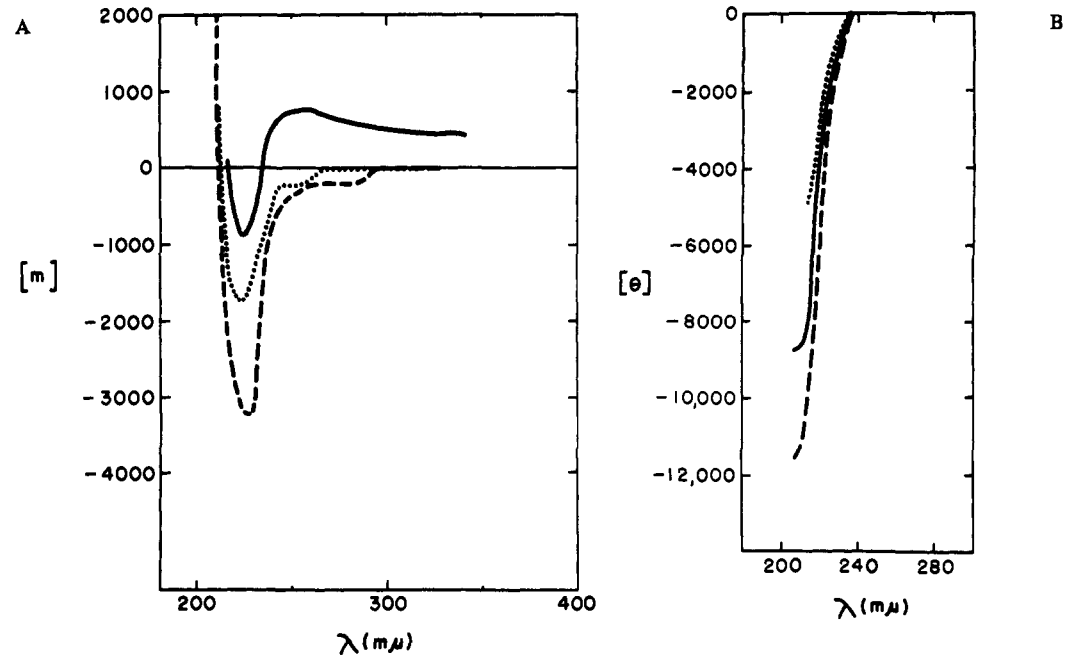


FIGURE 2: Optical rotatory dispersion and circular dichroism spectra of Yosizawa oligosaccharides. (A) Optical rotatory dispersion spectra. (—) β-D-GNAc-(1→3)-D-Gal; (·····) β-D-GNAc-(1→6)-D-Gal; (----) β-D-GNAc-(1→3)-[β-D-GNAc-(1→6)]-D-Gal. (B) Circular dichroism spectra. Same legend as in A.

it is not seen even at 211 mμ and probably occurs at 210 mμ or below. This may result from different degrees of overlap with the more intense $\pi$–$\pi^*$ positive band of the 2-acetamido group at still shorter wavelength (Listowsky *et al.*, 1968); from different perturbations on the acetamido group because of varying interactions of the group with other groups in the molecule; or from different extent of exposure to solvent of the acetamido group. Whatever the reasons, comparisons of the rotatory intensities of the transition may only be approximate when the relative rather than the maximum amplitudes are known.

With these qualifications stated, it is worthwhile to proceed to an examination of GNAc-containing di- and oligosaccharides. In Figures 1C and 2A,B are shown the optical rotatory dispersion and circular dichroism spectra of five disaccharides and one trisaccharide. In three of the disaccharides, the GNAc residues are equilibrium mixtures of the α and β anomers; in the other two and in the trisaccharide the GNAc residues are in β-glycosidic linkage to D-galactosyl residues. In the latter three, the Yosizawa oligosaccharides (Yosizawa, 1961), the optical rotatory dispersion spectra indicate additivity in that the sum of the $[m]_{\text{trough}} - [m]_{300\ m\mu}$ values from β-D-GNAc-(1→3)-D-Gal and β-D-GNAc-(1→6)-D-Gal equal the value for β-D-GNAc-(1→3)-[β-D-GNAc-(1→6)]-D-Gal.

The circular dichroism bands do not give an exactly comparable result. When, for example, the ellipticities at 215 mμ are compared, the trisaccharide value is lower than the sum of the two disaccharides, on a residue basis. However, the trisaccharide curve has not reached an extremum at the shortest wavelength shown and it is possible that additivity would be observed if the extrema were evaluated.

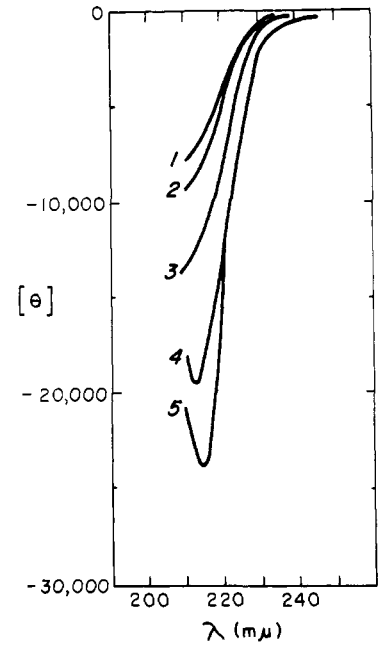


FIGURE 3: Circular dichroism spectra of the milk oligosaccharides. Curve 1: lacto-*N*-neotetraose; curve 2: lacto-*N*-tetraose; curve 3: lacto-*N*-fucopentaose II; curve 4: lacto-*N*-fucopentaose I; curve 5: lacto-*N*-difucohexaose I. The constituent residues and sequences of the milk oligosaccharides are shown in Table I.

In all three cases, the positions of the extrema are clearly shifted to shorter wavelengths with respect to Me-β-D-GNAc. Moreover the circular dichroism spec-

TABLE I: 2-Acetamido Cotton Effect and Circular Dichroism (Ellipticity) Band in Milk Oligosaccharides and Related Oligosaccharides.

| Symbol or Name | Optical Rotatory Dispersion $[m]_{trough} - [m]_{300}$ | Circular Dichroism $-[\theta]_{max}$ | $\lambda_{max}$ |
|---|---|---|---|
| β-D-Gal-(1→6)-D-GNAc | −1,240 | 4,840 | (218)[a] |
| β-D-Gal-(1→4)-D-GNAc | −2,940 | 5,000 | (218)[a] |
| β-D-Gal-(1→3)-D-GNAc | −4,550 | 5,360 | (218)[a] |
| Lacto-*N*-neotetraose | −3,140 | 7,500 | (212)[a] |
| Lacto-*N*-tetraose | −3,720 | 9,300 | 211 |
| Lacto-*N*-fucopentaose I | −7,040 | 19,200 | 212 |
| Lacto-*N*-fucopentaose II | −9,850 | 13,400 | 210 |
| Lacto-*N*-difucohexaose I | −11,500 | 24,200 | 214 |
| Lactodifucotetraose | −1,610 | No band | |

Lacto-*N*-neotetraose: β-D-Gal-(1→4)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-G

Lacto-*N*-tetraose: β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-G

Lacto-*N*-fucopentaose I: α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-G

Lacto-*N*-fucopentaose II: β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-G

4
↑
α-L-Fuc-1

Lacto-*N*-difucohexaose: α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-G

4
↑
α-L-Fuc-1

Lactodifucotetraose: α-L-Fuc-(1→2)-β-D-Gal-(1→4)-G

3
↑
α-L-Fuc-1

[a] No maximum recorded. Value in parentheses is shortest wavelength at which measurement could be made. Molecular ellipticity given for that wavelength.

trum reveals the intensification of the ellipticity band due to the β-linked GNAc residue wrought by substitution of the Gal residue for the methyl group of Me-β-D-GNAc, in the case of β-D-GNAc-(1→3)-D-Gal.

Figure 3 shows the circular dichroism spectra of the milk oligosaccharides. Values for molar rotations at 218 and 300 mμ have been published previously for these compounds. In Table I, the rotations are given as $[m]_{trough} - [m]_{300m\mu}$ and compared with the maximum intensities of the circular dichroism bands. As extrema were observed only with lacto-*N*-fucopentaose I and lacto-*N*-difucohexaose, the ellipticity values for the remaining milk oligosaccharides are reported at the shortest wavelengths at which measurements were made, as indicated in the table. The table also lists values for several other disaccharides. On the basis of the size of the Cotton effect troughs of these compounds and of other oligosaccharides whose rotations are given in previously published reports (Beychok and Kabat, 1965; Lloyd *et al.*, 1967, 1968), it is possible to arrange the oligosaccharides containing GNAc residues into three groups: (a) oligosaccharides in which the GNAc residue is β linked but unsubstituted; these compounds show the smallest Cotton effect troughs near 220 mμ (smaller than −2500 (deg cm$^2$)/dmole); (b) oligosaccharides which have their GNAc residues substituted either on C-3 or C-4 by β-D-Gal or α-L-Fuc-(1→2)-β-D-Gal; these compounds have intermediate values for $[m]_{trough} - [m]_{300m\mu}$ (between −3000 and −9000 (deg cm$^2$)/dmole); and (c) oligosaccharides in which the GNAc residue is disubstituted; these compounds have the largest corrected trough rotations (> −9000 (deg cm$^2$)/dmole).

As noted by Beychok and Kabat (1965) oligosaccharides in which the GNAc is substituted on C-3 tend to exhibit substantially greater n–π* Cotton effects than isomers with a C-4 substituent.

The circular dichroism spectra agree with the optical rotatory dispersion data ordering except in one instance: lacto-*N*-fucopentaose I exhibits a much more intense band near 210 mμ than does lacto-*N*-fucopentaose II, whereas the reverse is true in the optical rotatory dispersion spectra. This is a fairly serious discrepancy and has led us to reconsider even the approximate correctness of applying the $[m]_{trough} - [m]_{300m\mu}$ calculations for estimating the isolated

Cotton effect intensity when a fucosyl residue is included in the oligosaccharide. This in no way vitiates the validity of the grouping given above based on optical rotatory dispersion spectra. However, on the basis of the limited data available the isolated, or more nearly isolated, GNAc band in the circular dichroism spectra reveals that the optical activity of that group is more strongly affected by a disaccharide substituent than by a monosaccharide substituent on C-3. Furthermore, when the disaccharide substituent is on C-3, the GNAc ellipticity band is more intense than results from monosaccharide substituents on both C-3 and C-4, even when one of the monosaccharides is a fucosyl residue.

To gauge the effect of monofucosyl substitution on the GNAc Cotton effects and ellipticity bands, we have cast the data in a somewhat different form, as shown in Table II. Included in Table II are results with oligosaccharides derived from blood group substances (Lloyd *et al.*, 1967, 1968) as well as milk oligosaccharides. In each case, comparisons are made among a pair of oligosaccharides in which the only difference

TABLE II: Influence of Substitution of α-Fucosyl Residue on 2-Acetamido Cotton Effect or Circular Dichroism Band of β-Linked GNAc Residue

| Symbol or Name | Optical Rotatory Dispersion $[m]_{trough} - [m]_{300m\mu}$ | Circular Dichroism $[\theta]_{max}$ |
|---|---|---|
| | Substitution on C-3 | |
| B hexasaccharide ($BR_{IM5}$ 1.2)[a] | −8,400 | −11,100 |
| B pentasaccharide ($BR_L$ 0.44)[a] | −5,050 | −7,650 |
| | Δ = −3,350 | Δ = −3,450 |
| H pentasaccharide ($HR_{IM5}$ 2.5)[a] | −11,600 | −12,500 |
| H tetrasaccharide ($HR_L$ 0.75)[a] | −8,250 | −7,250 |
| | Δ = −3,350 | Δ = −5,250 |
| Lewis $R_L$ 0.71a[b] | −9,500 | −10,600 |
| Lewis $R_L$ 0.96 | −5,480 | −6,500 |
| | Δ = −4,020 | Δ = −4,100 |
| | Substitution on C-4 | |
| Lacto-*N*-fucopentaose II | −9,850 | −13,800 |
| Lacto-*N*-tetraose | −3,720 | −9,300 |
| | Δ = −6,130 | Δ = −4,500 |
| Lacto-*N*-difucohexaose I | −11,500 | −24,000 |
| Lacto-*N*-fucopentaose I | −7,040 | −19,500 |
| | Δ = −4,460 | Δ = −4,500 |

α-L-Fuc-1 (↓2), α-L-Fuc-1 (↓3)
$BR_{IM5}$ 1.2: α-D-Gal-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols

α-L-Fuc-1 (↓2)
$BR_L$ 0.44: α-D-Gal-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols

α-L-Fuc-1 (↓2), α-L-Fuc-1 (↓3)
$HR_{IM5}$ 2.5: β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols

α-L-Fuc-1 (↓2)
$HR_L$ 0.75: β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols

α-L-Fuc-1 (↓3)
Lewis $R_L$ 0.71a: β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols
Lewis $R_L$ 0.96: β-D-Gal-(1→4)-β-D-GNAc-hexenetetrols

[a] From Lloyd *et al.* (1967). [b] From Lloyd *et al.* (1968).

is the presence or absence of an L-fucosyl residue on C-3 or C-4 of a D-GNAc residue. The results are quite striking and reveal that the fucosyl substituent has a nearly uniform effect whether it is $\alpha$-(1→3) or $\alpha$-(1→4) linked. Furthermore, there is no discrepancy between the optical rotatory dispersion and circular dichroism results in any case, although included in the table are the two milk oligosaccharides which, when compared directly, yield apparently conflicting results in optical rotatory dispersion and circular dichroism spectra.

*Teichoic Acids.* The teichoic acids from *S. aureus* are polymers of ribitol phosphate in which 2-acetamido-2-deoxy-$\alpha$- and $\beta$-D-glucopyranosyl residues are linked to the polyol residues. In addition there is a small, but variable, number of D-alanine residues linked to the backbone. Different strains of *S. aureus* contain different proportions of $\alpha$- and $\beta$-linked GNAc residues in their teichoic acids (Baddiley *et al.*, 1962; Sanderson *et al.*, 1962; Nathenson *et al.*, 1966).

Figure 4 shows the optical rotatory dispersion spectra of four samples of teichoic acid. As discussed in the Materials section, the polymer from strain *3528* is known to have only $\alpha$-linked GNAc residues (Nathenson *et al.*, 1966); that from strain *NYH-6* was estimated immunochemically to be about 50% $\alpha$- and 50% $\beta$-linked GNAc (Torii *et al.*, 1964); that from strain *Copenhagen* comprises 15% $\alpha$- and 85% $\beta$-linked GNAc (Sanderson *et al.*, 1962); the polymer from strain *A1* was thought to contain only

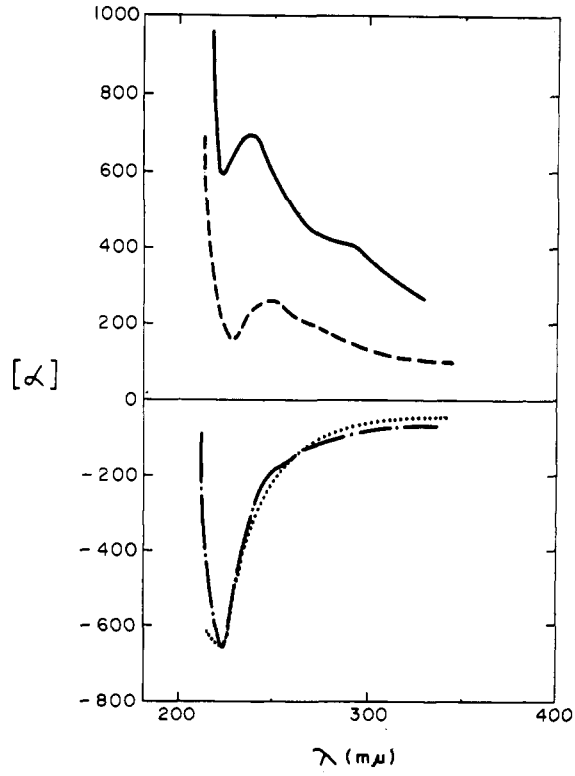


FIGURE 4: Optical rotatory dispersion spectra of four samples of teichoic acid from *S. aureus*. (—) strain *3528*; (-----) strain *NYH-6*; (·····) strain *Copenhagen*; (-·-·-) strain *A1*.

$\beta$-linked GNAc residues (Davidson *et al.*, 1964).

The optical rotatory dispersion spectrum of the teichoic acid from strain *3528* is quite similar to Me-$\alpha$-D-GNAc, and is thus largely dominated by the optical activity of the GNAc residue. The data are presented in terms of specific rather than molar rotation because of some uncertainty in the repeating residue weight. However, the approximate residue rotations may be obtained by multiplying the specific rotation by 4.55. This would then correspond to an $[m]_{300m\mu}$ value of about 1500 (deg cm$^2$)/dmole and may be compared to 1170 which is the value for Me-$\alpha$-D-GNAc. At 235 m$\mu$, $[m]$ for the polymer would be about 3190 which may be compared to 3100 for Me-$\alpha$-D-GNAc. This agreement should be taken as only semiquantitative, since the background rotation in the teichoic acid is sufficiently different from that of Me-$\alpha$-D-GNAc to cause a slight shoulder at 280 m$\mu$, which is absent in Me-$\alpha$-D-GNAc, and to bring about a sharper maximum at 235 m$\mu$ in the polymer than is shown by Me-$\alpha$-D-GNAc. Still, there is little question that $\alpha$-linked GNAc residues in the teichoic acid dominate the shape and largely scale the dispersion curve in this spectral interval.

The samples containing $\beta$-linked GNAc residues are slightly more difficult to evaluate. The teichoic acid from strain *Copenhagen* is known to contain 85% $\beta$-linked GNAc residues and 15% $\alpha$-linked residues. The shape of the curve is very close to that of Me-$\beta$-D-GNAc. At 300 m$\mu$, the approximate value of the molar rotation is $-185$ (deg cm$^2$)/dmole which is considerably more positive than the value of $-552$ given by Me-$\beta$-D-GNAc. At the trough at 220 m$\mu$, the value for the polymer is $-2915$ and $-2690$ (deg cm$^2$)/dmole for Me-$\beta$-D-GNAc. Again, considering the uncertainties involved, the agreement is quite good and the spectrum is obviously dominated by the GNAc residues.

The polymer from strain *A1* was stated to contain 100% $\beta$-linked GNAc residues and confirmatory evidence that this is the case has been obtained from its failure to precipitate with a purified snail hemagglutinin which reacts with $\alpha$-linked GNAc residues, while the Copenhagen strain reacts to an extent expected by the presence of 15% of $\alpha$-linked GNAc residues (S. G. Hammarström and E. A. Kabat, 1969, manuscript in preparation). Thus the optical rotatory dispersion measurements failed to detect the difference between 100 and 85% $\beta$-linked GNAc residues. The teichoic acid from strain NYH-6 gives a slightly more positive optical rotatory dispersion spectrum than might be expected for a polymer comprising equal numbers of $\alpha$- and $\beta$-linked GNAc residues and would be about 60% $\alpha$, but the approximations in the calculations and the uncertainty in the value for the fully linked $\beta$ polymer may account for this. It may be recalled in connection with all these samples that any D-alanine present would contribute negative rotation in the spectral region presented here.[2] While the influence of such

[2] In calculating the residue weight, we have assumed the presence of 0.5 residue of D-alanine/repeating unit (Nathenson *et al.*, 1966).

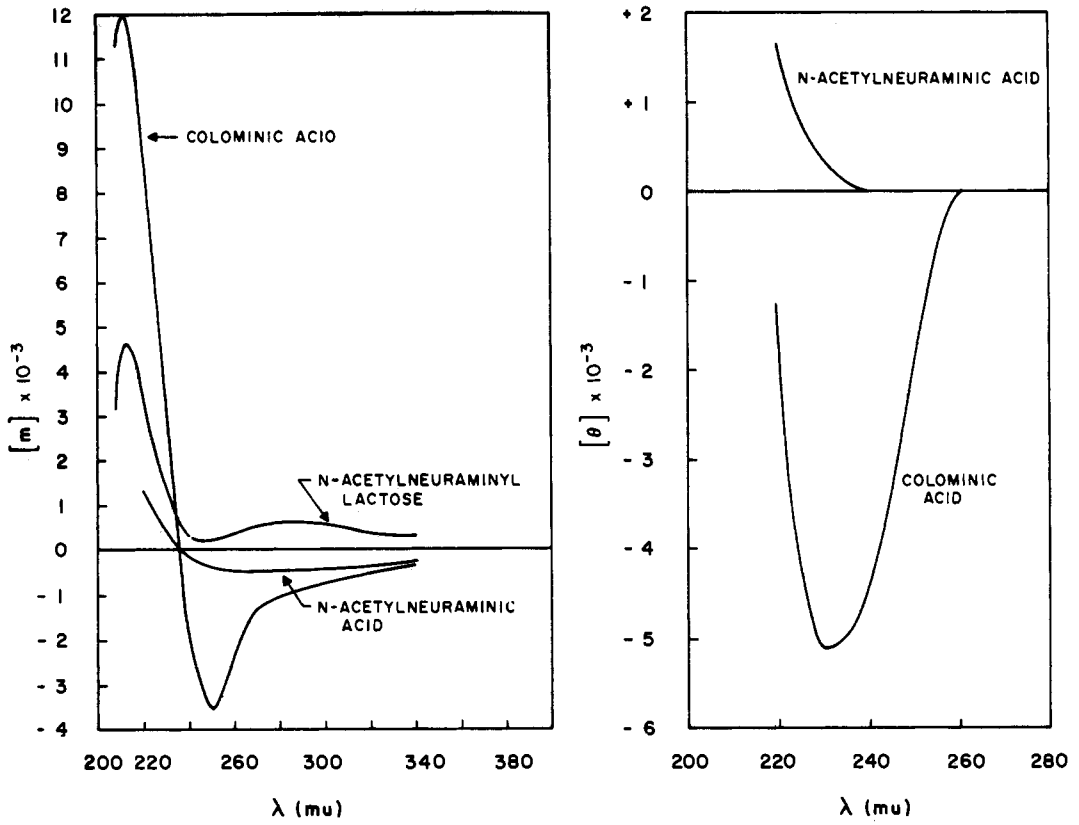


FIGURE 5: Optical rotatory dispersion and circular dichroism spectra of *N*-acetylneuraminic acid, *N*-acetylneuraminyllactose, and colominic acid. Left: optical rotatory dispersion spectra; right: circular dichroism spectra.

rotation is clearly small in all these spectra, it could necessitate a slight readjustment if quantitative agreement is sought between optical rotatory dispersion and other methods of determining relative amounts of α- and β-linked GNAc residues.

*N-Acetylneuraminic Acid, N-Acetylneuraminyllactose, and Colominic Acid.* The optical rotatory dispersion spectra of *N*-acetylneuraminic acid, *N*-acetylneuraminyllactose, and colominic acid are shown in Figure 5. The last compound is a polymer which yields only *N*-acetylneuraminic acid on hydrolysis (McGuire and Binkley, 1964). Circular dichroism spectra of *N*-acetylneuraminic acid and of colominic acid are also shown. The most noteworthy feature of both the optical rotatory dispersion and circular dichroism spectra is the profound difference between colominic acid and its constituent residues. We will comment below on these spectra. It may be noted here that an old sample of colominic acid perhaps hydrolyzed gave an optical rotatory dispersion spectrum very similar to that of *N*-acetylneuraminic acid.

## Discussion

It has thus far been possible to discern only a small number of quantitative or semiquantitative regularities in the optical rotatory dispersion and circular dichroism spectra of the GNAc-containing oligo- and polysaccharides. Among those that have thus far proved of great analytical value in structure determinations of the oligosaccharides of the blood group substances and several other biologically important compounds reported here and elsewhere are the following. (1) *Any substitution on a GNAc residue, particularly if the residue is β linked, brings about a very substantial intensification of the n–π* Cotton effect and associated circular dichroic (ellipticity) band.* In the latter, intensification is sometimes accompanied by and seen as a broadening of the band indicating a greater rotational strength for the transition (Beychok, 1966). Thus, one effect of substitution of a D-Gal residue on GNAc in β-D-Gal-(1→3)-D-GNAc, β-D-Gal-(1→4)-D-GNAc, and β-D-Gal-(1→6)-D-GNAc is an increased half-band width, although the maximum ellipticity is increased over that of GNAc by at least 1000 (deg cm$^2$)/dmole in all cases, as well, and the bands are displaced to longer wavelengths. The quantitative differences can only be calculated if the full circular dichroism band can be either isolated or estimated and, as noted in the Results section, this has not always been possible. (2) *As judged by the magnitude of the n–π* ellipticity band in the circular dichroism spectra, substitution of a disaccharide on C-3 of a β-linked GNAc residue results in a larger rotational strength for this GNAc transition than is brought about by any other substitution thus far examined.* The corresponding Cotton effect for a residue containing a disaccharide substituent on C-3 is very intense but somewhat less than that exhibited by a disubstituted GNAc residue when both substituents (on C-3 and C-4) are mono-

saccharides (one is an α-L-Fuc residue in all oligosaccharides we have thus far studied). This can be seen by comparing the values for lacto-*N*-fucopentaose I (−7040) and II (−9850). In the circular dichroism spectrum, the band is less intense for the residue disubstituted at C-3 and C-4 with monosaccharides as compared with those in which the GNAc residue is substituted on C-3 by α-L-fucosyl-(1→2)-D-galactose. Compare, for example, the $[\theta]_{max}$ values for lacto-*N*-fucopentaose II (−13,800) with lacto-*N*-fucopentaose I (−19,500). Part, or all, of this discrepancy may arise from the very steeply changing background in this spectral interval shown by GNAc residues substituted with fucose. Thus, the background is so steep in β-L-Fuc-(1→3)-D-GNAc that the GNAc Cotton effect is not seen at all (Figure 6). Even the circular dichroism curve of this compound is sufficiently overlapped with a strong, shorter wavelength band that it shows no clearly marked maximum. This background, or overlap, is obviously powerful enough to obscure the profound influence on the optical activity of the C-3 disaccharide substituted GNAc in the optical rotatory dispersion, but not in the circular dichroism spectra. (3) *In the circular dichroism and optical rotatory dispersion spectra, when analogous compounds are compared, substitution by an α-L-fucosyl residue leads to a large intensification (>3000 (deg cm²)/dmole) of the n–π* band.* Coincidentally the numerical values of the increase in $[m]_{trough} - [m]_{300m\mu}$ are very close to those of $[\theta]_{max}$, although it should be noted that an α-L-fucosyl residue is expected to contribute a substantial negative rotation to the optical rotatory dispersion value (Lloyd *et al.*, 1967). There is no marked difference in this intensification between C-3 and C-4 (α-L-Fuc) substitutions, although there is a marked difference in the optical activity of β-linked D-GNAc residues which are substituted by disaccharides on C-3 and C-4 (*cf.* Table II, Δ optical rotatory dispersion and circular dichroism values for pairs of oligosaccharides). (4) *Insofar as the n–π* transition of the 2-acetamido chromophore is concerned, the nature of the 2-acetamido-2-deoxy-D-glucosyl linkage (α or β) profoundly influences the shape, sign, and intensity of dispersion curves and significantly influences the intensity of the circular dichroism bands.* However, the substituent linked glycosidically to the GNAc appears not very important in the limited number of cases which we have tested. The teichoic acids offer a dramatic example of the latter insensitivity and the former sensitivity. In addition, the Yosizawa oligosaccharides provide additional evidence that β-linked, unsubstituted GNAc residues all appear to give similar values of $[m]_{trough} - [m]_{300m\mu}$, although the background rotation may differ considerably.

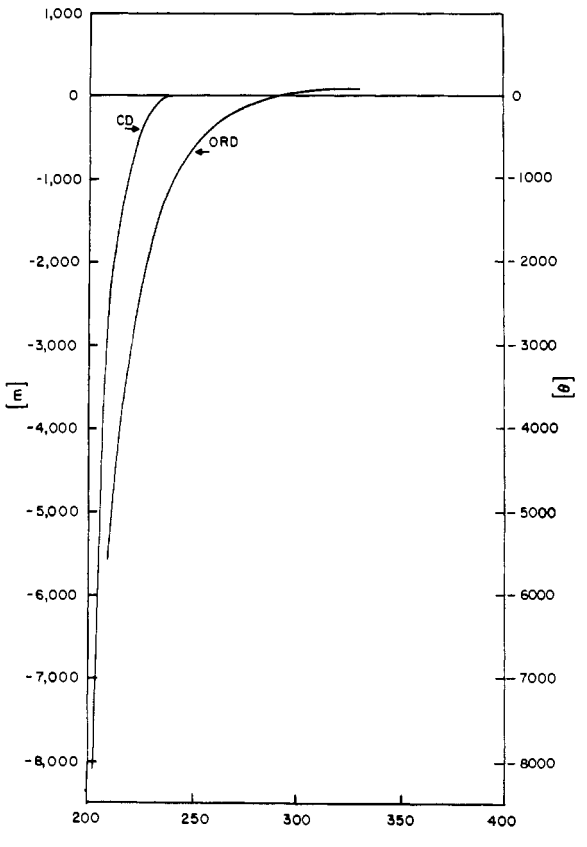


FIGURE 6: Optical rotatory dispersion and circular dichroism spectra of β-L-Fuc-(1→3)-D-GNAc.

In listing the above generalizations based on measurements of several dozen compounds, we are nevertheless aware of the limited numbers of observations thus far available. At present, the most serious difficulty is that circular dichroism maxima and band shapes are available for less than half of even those oligo- and polysaccharides for which partial circular dichroism spectra are given or have been previously presented.

*N-Acetylneuraminic Acid and N-Acetylneuraminyllactose.* *N*-Acetylneuraminic acid, as well as other sialic acids and their derivatives, are so widely represented in biological materials that it seems worthwhile to present as much data as possible. We restrict our brief comments to the spectacular differences between colominic acid and its constituent monomeric *N*-acetylneuraminic acid. In the polymer, no new chromophores are created except for a small number of hypothesized ester bonds (McGuire and Binkley, 1964). Most surprising is the extremely long wavelength of the Cotton effect trough at 250 mμ and the circular dichroism band maximum at 230 mμ. It may be noted that the peptide bonds of the α helix, which are completely shielded from solvent, generate an n–π* Cotton effect trough at the much shorter wavelength of 233 mμ; the random coil polypeptides exhibit a very small band near 240 mμ which is, as yet, unassigned (Beychok, 1966). Whatever the origin of this anomalous amide band, it would appear to be highly dependent on a particular conformation assumed by the polymer. Since it is unlikely that the ester bonds would generate a band at such a long wavelength, it must be assumed that the polymer folds in such a way as to involve the acetamido group in an interaction which strongly destabilizes the ground state. The group may very well be masked and inaccessible to solvent. Denaturation studies would appear to be profitable.

Finally, we should note, in reference to these substances, as well as to the teichoic acids, that the amounts necessary for examination of either the optical rotatory dispersion or circular dichroism spectra do not usually need to exceed 1.5 mg and, often, less will do. Considering the characteristic spectra, these measurements would seem to be highly valuable adjuncts to other analytical procedures.

Optical rotatory dispersion and circular dichroism measurements have proven of considerable aid in recognizing conformation of oligosaccharides in solution and provide an important independent line of evidence which, correlated with immunochemical studies, has made it possible to study the role of conformation in antigen–antibody interactions. While most of the correlations to date (Beychok and Kabat, 1965; Lloyd *et al.*, 1967, 1968) have been obtained with oligosaccharide determinants containing 2-acetamido groups, optical rotatory dispersion and circular dichroism measurements should prove applicable to the study of antigenic determinants of polypeptides and proteins. Thus, Crumpton and Small (1967) have found that a tetradecapeptide from myoglobin which in the intact molecule was predominantly helical but was nonhelical in aqueous solution nevertheless combined with antibody to metmyoglobin, suggesting perhaps, that the peptide was stabilized in the helical form in the antibody combining site. The finding of Schlossman *et al.* (1968) that the antibody combining sites of antibodies produced by injection of $\alpha$-DNP-$(Lys)_{11}$ were complementary to a determinant of the size of $\alpha$-DNP-$(Lys)_7$ while those produced by injection of $\alpha$-DNP-$(Lys)_{1200}$ were complementary only to $\alpha$-DNP-$(Lys)_3$ may be related to conformational differences between the two antigens (Kabat, 1966, 1968) and optical rotatory dispersion and circular dichroism studies might be beneficial in this connection.

From the standpoint of carbohydrate antigenic determinants, the optical rotatory dispersion measurements provided in a few hours estimates of the proportions of $\alpha$- and $\beta$-linked GNAc residues in staphylococcal teichoic acids which required extensive immunochemical study (Torii *et al.*, 1964). While the optical rotatory dispersion and circular dichroism studies cannot distinguish mixtures of $\alpha$- and $\beta$-teichoic acids from both linkages on a single polyribitol phosphate backbone, which is readily demonstrated immunochemically, they now provide a rapid estimate of the proportion of $\alpha$ and $\beta$ linkages in mixtures except when the proportion of $\alpha$ linkages is as low as in the Copenhagen strain.

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