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Optical Activity and Conformation of Carbohydrates. I. Optical Rotatory Dispersion Studies on Immunochemically Reactive Amino Sugars and Their Glycosides, Milk Oligosaccharides, Oligosaccharides of Glucose, and Blood Group Substances*

Sherman Beychok† and Elvin A. Kabat

ABSTRACT: Optical rotatory dispersion (ORD) spectra of a few selected carbohydrates and of the blood group A, B, and H substances are presented. The maltose and isomaltose series of oligosaccharides, the cyclic Schardinger dextrins, and amylose exhibit simple dispersion curves over the spectral region 589–200 mμ, with Drude dispersion wavelengths nearly the same for all but maltose. The blood group substances and Type XIV pneumococcal polysaccharide exhibit pronounced negative Cotton effects with troughs near 220 mμ. These Cotton effects are shown to be associated with the 2-acetamido (*N*-acetyl) group. The intensity of this Cotton effect is compared in a variety of 2-acetamido-2-deoxy sugars and in oligosaccharides containing *N*-acetylated sugars. The magnitude of the trough is about seventeen times greater in methyl β-(2-acetamido-2-deoxy)-D-glucoside than in ethyl β-(2-acetamido-2-deoxy)-D-galactoside. It is considerably diminished in the α anomers. Substitution of a β-D-galactosyl residue at carbon 3 or at carbon 4 in *N*-acetyl-D-glucosamine brings about an intensification of the trough magnitude. The deepest trough is observed with α-L-fucosyl-substituted *N*-acetyl-D-glucosamine as occurs in some of the milk oligosaccharides. An example of possible analytical utility is the requirement, from the ORD data, that the *N*-acetyl-D-glucosamine residues of Type XIV pneumococcal polysaccharide must be branched, in agreement with methylation studies of others.

Variations in the Cotton effect magnitude are interpreted using the octant rule and assuming a preferred orientation of the planar amide group with respect to the ring. The significance of these results is discussed, especially with respect to the relationship of preferred conformations of sugar rings to immunochemical specificity and antibody heterogeneity.

In the 148 years since Biot's discovery of the optical activity of cane sugar (Biot, 1817), a great number of measurements have been made of the optical rotation of carbohydrates. The majority of these were performed at a single wavelength (sodium D line or mercury green line). Attempts to understand the measured specific rotations in terms of the chemical structure have depended largely on the use of empirical rules such as those of van't Hoff (1875) and Hudson (1909). Lowry (1935) has reviewed the very considerable literature which had accumulated by that date. More recently, Kauzmann *et al.* (1961) have formulated rules for predicting the rotation of simple carbohydrates based on a summation of pairwise interactions.

This paper is concerned with the optical rotatory dispersion (ORD)[1] of a few selected carbohydrates and of the blood group A, B, and H substances to determine whether ORD reveals anything about ordered or periodic structures in polymeric carbohydrates and also

* From the Departments of Biochemistry, Microbiology, and Neurology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, Presbyterian Hospital, New York, N. Y. *Received June 21, 1965; revised August 13, 1965.* This work was aided by grants from the United States Public Health Service (GM 10576), the National Science Foundation (GB 1913, G 18727, and GB 3675), the Office of Naval Research, and the General Research Support Grant, National Institutes of Health, United States Public Health Service.

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[1] Abbreviations used in this work: ORD, optical rotatory dispersion; GNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; G, D-glucose, Gal, D-galactose.

to evaluate the influence on the rotatory dispersion spectra of certain substituents in mono- and oligosaccharides. It was considered of interest to ascertain whether dispersion curves of the maltose series of oligosaccharides and of amylose would reveal changes of structure with increasing size of the oligomer. Whether amylose is a helical polymer in solution is anything but settled (Rao and Foster, 1963). It was hoped that ORD measurements on oligosaccharides of the maltose series (α-1,4) would provide structural information which might be correlated with immunochemical findings. Thus in studying the inhibition by oligosaccharides of the precipitin reaction of dextran with human antidextran of α-1,4 specificity, it was found that inhibition by maltotriose, -tetraose, and -pentaose was greater than that by maltose on a molar basis, but that maltoheptaose was less effective than the tri-, tetra-, or pentasaccharides. This finding was interpreted on the hypothesis that since maltoheptaose could form a single turn of a helix, the effective concentration of the open chain form which reacted with antibody to cause inhibition was reduced (Kabat, 1954). Direct evidence for the existence of a helical form could provide strong support for this hypothesis.

The blood group A, B, H, and Le^a substances contain about 20–25% of amino acids (Carsten and Kabat, 1956; Pusztai and Morgan, 1963, 1964) in addition to L-fucose, D-galactose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine (*cf.* Kabat, 1956; Morgan, 1960). Apart from evidence indicating linkage of oligosaccharide side chains to serine and threonine (Anderson *et al.*, 1964a,b; Kabat *et al.*, 1965) probably by an *N*-acetyl-D-galactosamine (Kabat *et al.*, 1965), little is known of the over-all structure of the molecule or of the linkages of the amino acids to one another. It was possible that ORD spectra could provide a clue to some aspects of the structure of blood group substances.

As will be shown below, all the blood group substances exhibit negative Cotton effects with troughs near 220 mμ. These are due predominantly to the 2-acetamido-2-deoxyhexose moieties. Similar Cotton effects occur in 2-acetamido-2-deoxyglucose-containing oligosaccharides from human milk, which in many respects are similar to blood group oligosaccharides (Kuhn, 1957, 1958; Egge, 1960; Montreuil, 1960; Kuhn and Gauhe, 1962), as well as in simple *N*-acetylated amino sugars. It is quite likely that these Cotton effects will be useful in assessing structural features in polysaccharides containing significant quantities of *N*-acetylated sugars. We shall describe in some detail their magnitudes and signs in *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, their glycosides, and polymers containing these residues, such as blood group substances, oligosaccharides from human milk, and Type XIV pneumococcal polysaccharide.

Materials

The isomaltose series of oligosaccharides from isomaltose to isomaltoheptaose were prepared in this laboratory (Schlossman and Kabat, 1962) by the procedure described by Turvey and Whelan (1957). Samples of isomaltose and isomaltotriose were also made available by Dr. A. Jeanes. Maltose was supplied by Dr. D. P. Langlois, maltotriose by Dr. K. Wallenfels, maltotetraose by Dr. J. H. Pazur, maltopentaose by Dr. R. L. Whistler, maltoheptaose (two samples) by Drs. D. French and J. H. Pazur, maltooctaose, -nona-, and -decaose by Dr. W. J. Hoover, cyclohepta- and cyclooctaamylose from Dr. D. French, cyclopenta- and cyclohexaamylose and a second sample of cycloheptaamylose from Dr. O. Westphal.

D-Glucosamine, D-galactosamine, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine; GNAc), 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine; GalNAc), D-glucose (G), and D-galactose (Gal) were available in the laboratory. Methyl α- and β-glycosides of GNAc and methyl α-GalNAc were obtained from Professor R. Kuhn; ethyl β-GalNAc was obtained from Dr. R. Jeanloz. Methyl α-D-glucopyranoside was from Bios Laboratories; methyl β-D-glucopyranoside and methyl α- andβ-D- galactopyranosides were from Dr. N. K. Richtmyer. *O*-β-D-Galactopyranosyl-(1→3)- and -(1→4)-2-acetamido-2-deoxy-D-glucoses (β-Gal-(1→4)-GNAc and β-Gal-(1→3)-GNAc) were from Dr. F. Zilliken, and the corresponding β(1→6) disaccharide was from Professor R. Kuhn; the α(1→6) disaccharide was from Drs. W. M. Watkins and W. T. J. Morgan. The oligosaccharides from human milk, lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaoses I and II, lacto-*N*-difucohexaose I, and lactodifucotetraose were supplied by Professor R. Kuhn (Kuhn, 1957, 1958; Kuhn and Gauhe, 1962, Egge, 1960); their exact chemical structures are given in Table IV. All sugars are of the D series except L-fucose, and all are in the pyranose form.

Blood Group Substances

Samples of blood group A substances from human ovarian cyst, MSM 10%, and McDon were those described previously (Schiffman *et al.*, 1964). The other samples of MSS were from a new batch prepared from alcohol-precipitated ovarian cyst fluid. MSS phenol insoluble, MSS 10%, MSS phenol insoluble from first 10% precipitate, and a sample of MSS 10% which had been thoroughly dialyzed were of comparable analytical properties; the blood group A activity of the phenol-insoluble fractions was less than that of the 10% precipitates. Cyst 9 was a sample of blood group A substance kindly supplied by Dr. Harold Baer. Alcohol precipitates of ovarian cyst fluids, cyst 14 (A_2) and cyst 19 (B), were also from Dr. Baer. These were purified in the usual manner (Kabat, 1956) and phenol-insoluble and 10% precipitates were obtained from each. An additional sample of blood group B substance Beach, phenol insoluble, and a sample of H substance J.S. (group O), were also described previously (Schiffman *et al.*, 1964). A second sample of H substance Tighe (group O) was prepared in this laboratory by Dr. Donald Marcus. All samples were subjected to prolonged dialysis before final isolation. Solutions were

prepared in water from samples dried to constant weight over P_2O_5 at room temperature. Type XIV pneumococcal polysaccharide was the sample prepared in this laboratory (Howe *et al.*, 1958).

Methods

Optical rotatory dispersion measurements were performed on a Bendix-Ericcsen automatic recording spectropolarimeter, Model Polarmatic 62. The instrument is equipped with a double prism monochromator (Gillham and King, 1961). The spectral range of the instrument is 186–667 mμ, although the measurements recorded here were possible only to 198 mμ. Representative values for half-band widths at different slit settings have been reported elsewhere (Beychok, 1964). In the spectral region 546–230 mμ measured rotations were reproducible to ±0.0005°. Below 230 mμ the precision diminished, and at 200 mμ the reproducibility was ±0.003°. In most cases, this represented 2% or less of the total measured rotation. Calibrated cylindrical silica cells of 1.000, 0.500, 0.109, and 0.0167 cm were used. All measurements were made at room temperature which was maintained between 22 and 24°.

Rotations are expressed as specific rotations or, alternatively, as molecular rotations when the mean monomer weights are known.

$$[\alpha]_\lambda = \frac{\alpha_{obsd}}{lc}$$

$$[m]_\lambda = [\alpha]_\lambda \times \text{mol wt}/100 \qquad (1)$$

in which $[\alpha]_\lambda$ is the specific rotation at wavelength λ, $[m]_\lambda$ is the molecular rotation, l is the path length in decimeters, c is the concentration in grams/milliliter, and mol wt is the mean monomer molecular weight.

Results

In Figure 1 are shown the optical rotatory dispersion curves of the maltose series of oligosaccharides from the dimer (m_2) to the heptamer (m_7). The dispersion curves are all simple throughout the entire spectral region examined from 589 to 200 mμ. There is no indication, in the far ultraviolet, of a Cotton effect for any of these. Similar behavior is exhibited by amylose, itself, in water–dimethyl sulfoxide (56:44, v/v), by the isomaltose series from the dimer to heptamer, and by the cyclic Schardinger dextrins from the pentamer through the octamer. Additional evidence that no Cotton effects are encountered to wavelengths as short as 200 mμ is available from the observation that the dispersions for all the substances listed above may be fitted by single term Drude equations:

$$[m]_\lambda = \frac{K}{\lambda^2 - \lambda_c^2} \qquad (2)$$

in which K and λ_c are constants. In Figure 2 are shown

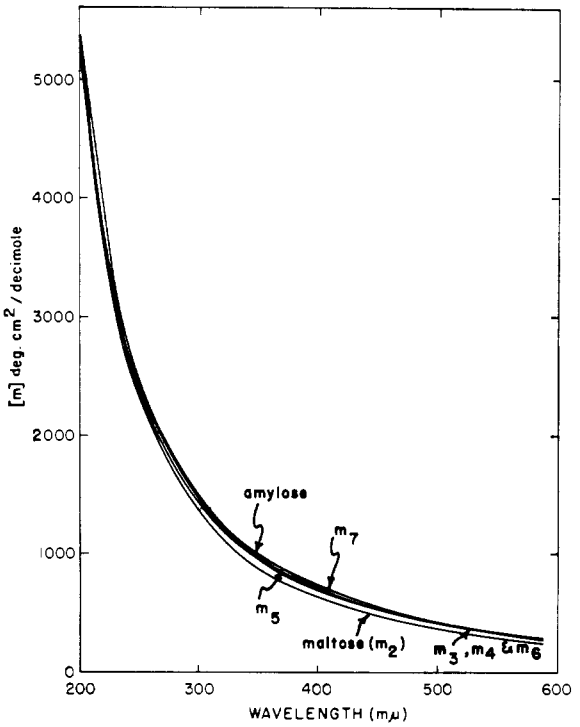

FIGURE 1: ORD of the maltose series of oligosaccharides (m_2 – m_7) in aqueous solution and of amylose in H_2O–DMSO (56:44, v/v).

the plots of $[m]_\lambda\lambda^2$ *vs.* λ^2 (linear, if eq 2 is obeyed) for the maltose series of Figure 1 but extended to the decamer. Without exception, a single term Drude equation accommodates the dispersion for each oligomer to 200 mμ.

Table I lists values of λ_c for the maltose and isomaltose oligosaccharides, several Schardinger dextrins, and amylose. The most notable feature of the table is that, with the exception of maltose itself, there is constancy of λ_c values within experimental error, including that observed with amylose in water–dimethyl sulfoxide. In the latter case, solvent absorption prevented measurements below about 235 mμ, but there was no deviation from eq 2.

Blood Group Substances

The initial objective in the examination of the ORD of the blood group substances was to see whether any ordered structures could be detected in the peptide portions of these materials. Since the blood group substances examined contain 20–25% by weight of amino acids (Carsten and Kabat, 1956; Pusztai and Morgan, 1963, 1964) it appeared worthwhile to investigate the possibility of occurrence of small segments of α helix. Because of the contribution to the rotation throughout the visible spectrum by the carbohydrate moieties of the blood group substances, a conventional analysis of the ORD (Moffitt and Yang, 1956) is not possible. In recent years, however, conformation-dependent Cotton effects, due to peptide

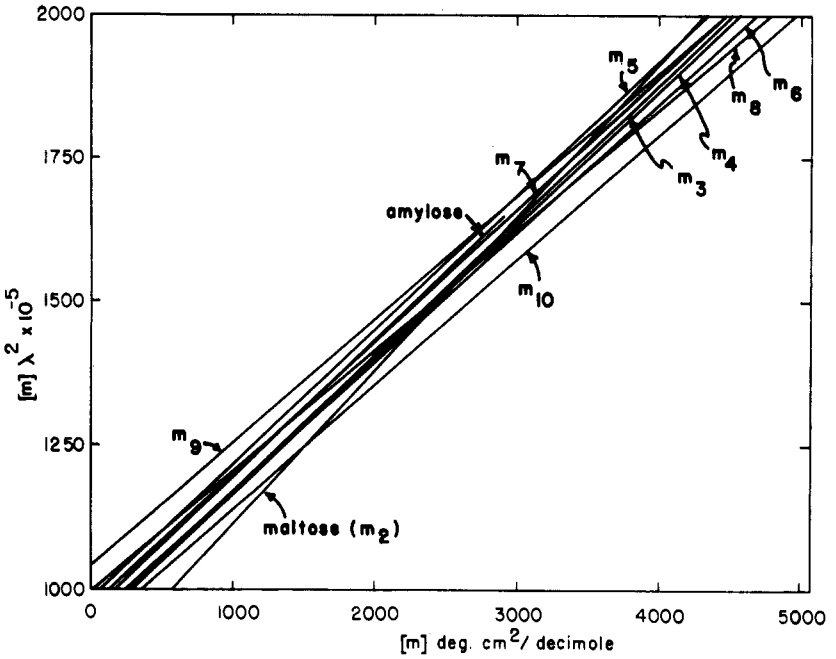

FIGURE 2: Drude plots of the data of Figure 1 for m_2 — m_7 and for amylose, as well as for m_8 — m_{10}.

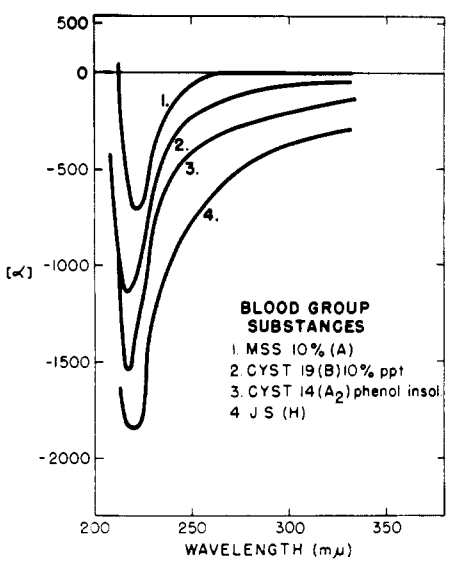

FIGURE 3: Far-ultraviolet ORD of four blood group substances in aqueous solution.

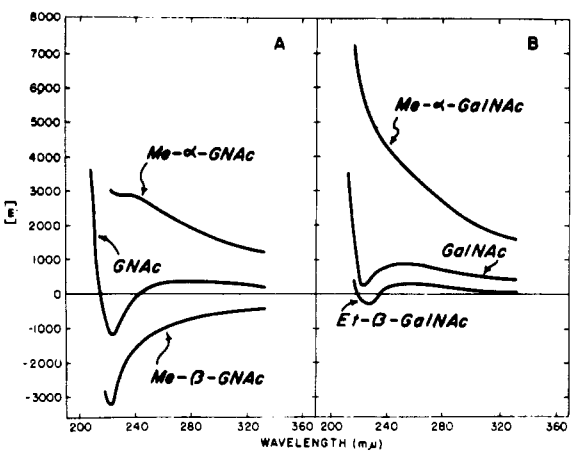

FIGURE 4: ORD curves of GNAc (A) and GalNAc (B) and their α- and β-glycopyranosides in aqueous solution.

absorption bands, have been discovered in the far ultraviolet. In polypeptides and proteins, these Cotton effects serve to characterize both the sense and quantity of α helix (Simmons *et al.*, 1961; Beychok and Blout, 1961; Blout *et al.*, 1962). With right-handed, α-helical polypeptides a very substantial trough of a Cotton effect occurs at 233 mμ. The *in vacuo* residue rotation is of the order of $-15{,}000°$ at 233 mμ. The inflection point of this Cotton effect occurs at about 225 mμ, below which wavelength the rotation increases in a positive direction reaching a value of about 65,000° at 200 mμ. In the random coil form of these polypeptides, a Cotton effect is observed at about 200 mμ (Blout *et al.*, 1962). The trough at 233 mμ can be used to detect relatively low content of helix in proteins. In view of the simple dispersion observed for the homo-oligo- and polysaccharides in this spectral region, it was anticipated that even a small peptide bond Cotton effect would be readily discerned.

In Figure 3 are shown the ORD curves of representative samples of blood group substances. There is, in fact, an unmistakable Cotton effect in all. The wave-

TABLE I: Drude Dispersion Constants.

Substance	λ_c (mμ)
Isomaltose (IM_2)	156 ± 2[a]
Isomaltotriose (IM_3)	154 ± 1
Isomaltotetraose (IM_4)	154 ± 1
Isomaltopentaose (IM_5)	154 ± 1
Isomaltohexaose (IM_6)	154 ± 1
Isomaltoheptaose (IM_7)	155 ± 1
Maltose (M_2)	163 ± 3
Maltotriose (M_3)	151 ± 1
Maltotetraose (M_4)	151 ± 1
Maltopentaose (M_5)	152 ± 2
Maltohexaose (M_6)	150 ± 1
Maltoheptaose (M_7)	149 ± 3
Maltooctaose (M_8)	145 ± 2
Maltononaose (M_9)	147 ± 4
Maltodecaose (M_{10})	147 ± 3
Amylose[b]	153 ± 1
Cyclopentaamylose	156 ± 3
Cyclohexaamylose	148 ± 4
Cycloheptaamylose	148 ± 3
Cyclooctaamylose	154 ± 3

[a] Range of deviation of averages from separate independent measurements. [b] In water–dimethyl sulfoxide (56:44, v/v).

lengths at which the troughs and inflection points are found, however, do not correspond to those described above for the polypeptides. Instead, the troughs occur at 218–223 mμ and the inflection points are near 210 mμ. These parameters are incompatible with amino acid residues in the hydrogen-bonded α helix or in the random coil. Table II lists the values of trough rotations observed in all the blood group substances thus far examined.

In view of the presence in these blood group substances of substantial quantities of GNAc and GalNAc (*cf.* Kabat, 1956; Morgan, 1960) it was thought that the 2-acetamido (*N*-acetyl) substituent on an optically active carbon might be the source of this Cotton effect. This group contains an amide bond, and there are two well-known electron transitions of the amide bond in this spectral region (Peterson and Simpson, 1957).

Figure 4A shows the ORD of 2-acetamido-2-deoxy-D-glucose and its methyl α- and β-glycopyranosides in the ultraviolet; Figure 4B shows the ORD of 2-acetamido-2-deoxy-D-galactose, its methyl α- and ethyl β-galactopyranosides. Because of instrumental limitations and high absorbancy it was possible to demonstrate an inflection point for only one of these substances, but the trough of a negative Cotton effect is clearly discerned in all but two of the spectra.[2] It is interesting to note the dramatic difference in the trough depths brought about by change of configuration about carbon 4 in the β anomers. Furthermore, Me α-GNAc shows only a barely discernible trough at wavelengths corresponding to the trough observed in the β form, and Me α-GalNAc exhibits no discernible Cotton effect at all. In Table III the rotations of the sugar glycosides are compared with the corresponding glycosides of the 2-acetamido-2-deoxy sugars. On a molar basis the difference in trough rotation between the Me β-G and Me β-GNAc is about seventeen times as great as the corresponding differences between Me β-Gal and Et β-GalNAc. The same kind of comparison for the α anomers indicates that the contribution of the 2-acetamido substituent is still negative for the α-glycoside of GNAc. Within experimental error, there is no difference in rotation at the trough wavelength between Me α-Gal and Me α-GalNAc. The effect of 2-acetamido substitution on the ORD spectrum in this region is thus small in the β-galactopyranoside and absent in the α-galactopyranoside. The effect of the acetamido on C-2 in both the α- and β-D-glucopyranosides is marked, giving rise to an unmistakable negative Cotton effect in the latter and adding a substantial negative rotation in the former.

These observations are extended in Table IV in which are listed rotations at troughs of compounds in which the GNAc residue is linked with various substituents in several positions. Of particular interest are the human milk oligosaccharides which are very similar to sequences in the blood group substances and Type XIV pneumococcal polysaccharide.

Except in the cases of lactodifucotetraose, which has no GNAc residue, and of α-Gal-(1→6)-GNAc, all the substances exhibit a Cotton effect trough between 218 and 222 mμ, characteristic of the GNAc moiety. GNAc, at equilibrium in solution, is about 60% α anomer. If the equilibrium is not displaced by an α-(1→6)-galactosyl substitution, then the expected rotation at 218 mμ would be about 4200 deg cm² per dmole. The observed value is actually greater by about 1270 deg cm² per dmole, which could be due to altered equilibration of α and β anomers. The same may be true for the β-(1→6)-galactosyl substitution, but for the β-Gal-(1→4)-GNAc the equilibrium mixture would have to be about 98% β anomer, which seems unlikely; for the β-Gal-(1→3)-GNAc the measured value is too negative by 1400 deg cm² per dmole even assuming 100% β anomer.

In comparing the magnitude of the 2-acetamido Cotton effect in different substances, we are very much hampered by an inability to cope with a background curve due to all the other substituents in the molecules. For this reason in Table IV (and Table III, as well) are listed also the rotations at 300 mμ as an indication, albeit an extremely crude one, of changes in the ORD not primarily due to changes in the magnitude of the 2-acetamido Cotton effect. This approximation is a very poor one for the equilibrium mixtures and the β anomer of galactose which exhibit complex dispersion

[2] In a recent communication, Pace *et al.* (1964) remark that *N*-acetyl-D-glucosamine exhibits a Cotton effect in the 220 mμ region of the spectrum.

TABLE II: Far-Ultraviolet Rotational Parameters of Blood Group Substances.

Sample	$[\alpha]_{trough}$	$[\alpha]_{300}$	$[\alpha]_{trough} - [\alpha]_{300}$	Hexosamine (%)
A substances				
MSS 10%	−725	+55	−780	33
MSS 10% dialyzed	−695	0	−695	35
MSS phenol-insol, 1st 10% ppt	−750	+245	−995	35
MSS phenol-insol	−985	+10	−995	32
MSM 10%	−840	−10	−830	32
McDon	−925	0	−925	33
Cyst 9	−485	+5	−490	30
A_2 substances				
Cyst 14 10%	−1740	−240	−1500	
Cyst 14 phenol insol	−1545	−210	−1335	26
H substances				
Tighe	−1700	−325	−1375	
J.S.	−1845	−370	−1475	24
B substances				
Cyst 19 10%	−1140	−55	−1085	27.5
Cyst 19 pheno-insol	−1175	−125	−1050	29
Beach phenol-insol	−1035	0	−1035	25

TABLE III: Characteristics of 2-Acetamido Cotton Effect.

Substance	Mol Wt	$[m]_{trough}$	$[m]_{300}$	$[m]_{trough} - [m]_{300}$
Me α-GalNAc	235	5360[a]	2060	3300
Me α-Gal	194	5330[a]	2120	3210
		30	−60	+90
Et β-GalNAc	249	−336	+134	−470
Me β-Gal	194	−225[a]	−90	−135
		−111	+224	−335
Me α-GNAc	235	3100	1170	1930
Me α-G	194	3940[a]	1670	2270
		−840	−500	−340
Me β-GNAc	235	−2690	−552	−2140
Me β-G	194	−775[a]	−321	−454
		−1915	−231	−1685

[a] $[m]$ at wavelength corresponding to trough. Me = methyl, Et = ethyl.

curves in the near ultraviolet (Listowsky *et al.*, 1965). Glucose shows no such complexity.

In comparing the magnitude of the trough exhibited by lacto-*N*-tetraose with that shown by β-Gal-(1→3)-GNAc it seems reasonable that the increased positive rotation at 300 mμ is due to the added lactose and that comparison of $[m]_{218} - [m]_{300}$ values is somewhat more realistic than comparison of trough rotations. When examined in this fashion, the differences between lacto-*N*-tetraose and lacto-*N*-neotetraose (−580 deg cm^2 per dmole) are not readily ascribed to differences in background rotation. Lactodifucotetraose is devoid of a

TABLE IV: Influence of Substitution on 2-Acetamido Cotton Effect.

Substance	Trough wavelength (mμ)	Mol Wt	$[m]_{218}$	$[m]_{300}$	$[m]_{218} - [m]_{300}$
α-Gal-(1→6)-GNAc	No trough	383	5470	2710	2760
β-Gal-(1→6)-GNAc	218–222	383	−860	+380	−1240
β-Gal-(1→4)-GNAc	218	383	−2860	+80	−2940
β-Gal-(1→3)-GNAc	218	383	−4440	+110	−4550
Lacto-*N*-neotetraose	218–220	707	−2510	+575	−3140
Lacto-*N*-tetraose	218	707	−3140	+580	−3720
Lacto-*N*-fucopentaose I	224	853	−3330	−520	−2810
Lacto-*N*-fucopentaose II	218–222	853	−8790	−1330	−7460
Lacto-*N*-difucohexaose I	222	999	−9390	−2930	−6460
Lactodifucotetraose	No trough	634	−2450	−840	−1610

Lacto-*N*-neotetraose: *O*-β-Gal-(1→4)-*O*-β-GNAc-(1→3)-*O*-β-Gal-(1 → 4)-G
Lacto-*N*-tetraose: *O*-β-Gal-(1→3)-O-β-GNAc-(1→3)-*O*-β-Gal-(1→4)-G
Lacto-*N*-fucopentaose I: *O*-α-Fuc-(1→2)-*O*-β-Gal-(1→3)-*O*-β-GNAc-(1→3)-*O*-β-Gal-(1→4)-G
Lacto-*N*-fucopentaose II: *O*-β-Gal-(1→3)-*O*-β-GNAc-(1→3)-*O*-β-Gal-(1→4)-G
↑ 4, 1
α-Fuc
Lacto-*N*-difucohexaose: *O*-α-Fuc-(1→2)-*O*-β-Gal-(1→3)-*O*-β-GNAc-(1→3)-*O*-β-Gal-(1→4)-G
↑ 4, 1
α-Fuc
Lactodifucotetraose: *O*-α-Fuc-(1→2)-*O*-β-Gal-(1→4)-G
↑ 3, 1
α-Fuc

GNAc residue and exhibits no Cotton effect in this spectral region. The rotations at 218 and 300 mμ and the difference between the two is about what one would expect for addition of two α-fucosyl residues to lactose. Again, in lacto-*N*-fucopentaose I, the α-(1→2)-fucosyl addition to the nonreducing galactose of lacto-*N*-tetraose is reflected in the negative rotation at 300 mμ, but the trough rotation shows no increased negativity and the Cotton effect has thus been diminished in intensity. When the α-(1→4)-fucosyl residue is substituted directly on the GNAc moiety there is a pronounced increase in the value of $[m]_{218} - [m]_{300}$, amounting to about −3500 deg cm² per dmole in both lacto-*N*-fucopentaose II and lacto-*N*-difucohexaose. The milk oligosaccharide studies thus indicate that substitutions by β-D-galactopyranosyl at carbons 3 and 4 and by α-L-fucopyranosyl at carbon 4 result in pronounced intensification of the 2-acetamido Cotton effect.

Figure 5 shows the far-ultraviolet ORD of Type XIV pneumococcal polysaccharide, a polymer consisting of D-glucose, D-galactose, and *N*-acetyl-D-glucosamine. The arrangement of residues is not known, but sequences of the type *O*-β-Gal-(1→4)-*O*-β-GNAc-(1→3)-Gal and *O*-β-G-(1→4)-*O*-β-GNAc-(1→3)-Gal occur often, with β-D-Gal-(1→6)-branching substitution on the GNAc residues likely from methylation studies (Barker *et al.*, 1958). If the data shown in Figure 5 are converted to molar rotations using the residue weight of

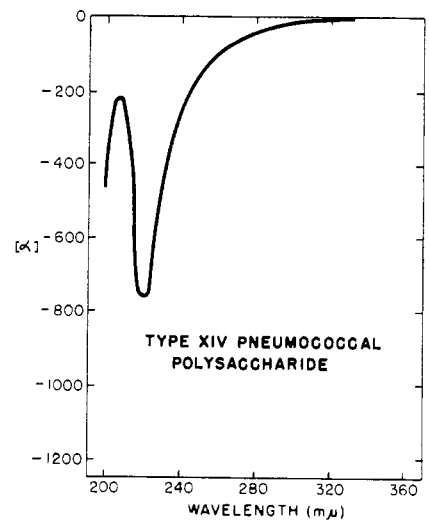

FIGURE 5: Far-ultraviolet ORD curve Type XIV pneumococcal polysaccharide in aqueous solution.

lacto-*N*-neotetraose, then $[m]_{218} - [m]_{300}$ is about −5100 deg cm² per dmole. This value seems too negative relative to lacto-*N*-neotetraose to be explained by the occasional occurrence of GNAc substituted by *O*-β-D-glucopyranosyl units, but we have made no measurements on compounds containing such a se-

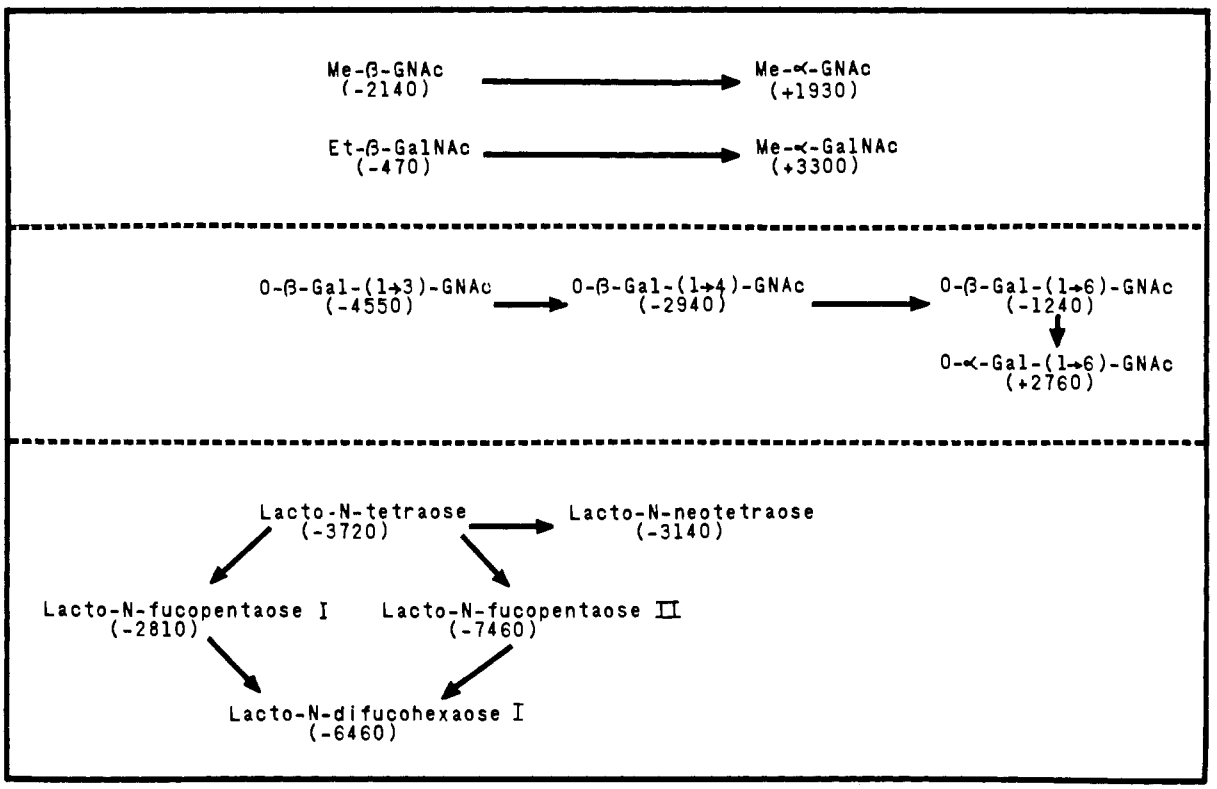

FIGURE 6: Schematic representation of changes in molar rotation ($[m]_{218} - [m]_{300}$) accompanying structural changes among oligosaccharides.

quence. The magnitude of the trough certainly appears to rule out linear sequences similar to the first three residues of lacto-*N*-neotetraose without large numbers of branch points at the GNAc residues, in accord with the methylation studies of Barker *et al.* (1958).

Discussion

Maltose and Isomaltose Oligosaccharides and Amylose

In the solid state, amylose can exist as a helical polymer, free or in complex with iodine or butanol (Rundle and French, 1943; Rundle and Edwards, 1943). Whether amylose may also assume a helical conformation in solution is uncertain, although Rao and Foster (1963) have presented hydrodynamic and optical evidence which leads them to suggest that the helical conformation is likely. Our optical rotatory dispersion results militate somewhat against a helical conformation, at least in the solvent system used. (With regard to solvent, Rao and Foster found no difference in ORD of amylose in DMSO and neutral aqueous solution with a λ_c for both of 148 mμ. This is slightly lower than our mean value of 152 mμ, but the difference is almost within our range of uncertainty.) The ORD results, however, cannot exclude a helical polymer for at least two important reasons. The first is that the geometry of the helix in the solid state allows relatively little interaction among chromophores of the type necessary to produce complex dispersion (Moffitt, 1956). The helix may be described as very loose. The distance along a turn, which comprises 6 glucose units, is about 8 A, and there is little ring overlap or stacking. We would expect relatively weak interaction among the light-induced dipoles in the hydroxyls, which are all equatorial, and consequent weak splitting of the absorption bands (necessary for complex dispersion). In the absence of such band splitting a single-term Drude may be expected to fit the ORD data.

Second, a distinct screw sense might exist in a molecule even as small as the maltotriose which may have as much dissymmetry built in as the longer oligomers. That the Schardinger dextrins give rise to the same dispersion constants as the oligo- and polysaccharides adds plausibility to this possibility. Thus the ORD data neither confirm nor disprove the inference from the immunochemical studies that maltoheptaose might exist in helical form in solution (Kabat, 1954).

2-Acetamido Cotton Effect

The scheme shown in Figure 6 summarizes several of the more important substitutions on GNAc from the point of view of increase or decrease in $[m]_{218} - [m]_{300}$, *i.e.*, alterations in the 2-acetamido Cotton effect. This Cotton effect is probably the $n-\pi^*$ transition of the amide group in which a nonbonding oxygen is promoted to an antibonding molecular orbital involving the oxygen and carbon (Kasha, 1961). The assignment is not absolutely secure because no solvent tests have been

made, but it is reasonable because the inflection wavelength (between 208 and 213 mμ) is rather high for the $\pi-\pi^*$ transition; the absorption intensity near 210 mμ is low for the electrically allowed $\pi-\pi^*$ transition and reasonable as end absorption which includes the electrically forbidden $n-\pi^*$ transition.

There is now abundant experimental evidence that the effect of various substituents on the ORD of ketones in an asymmetric environment can be predicted by application of the octant rule, provided the molecular framework is rigid (Moffitt *et al.*, 1961). Because of the substantial magnitude of the Cotton effects exhibited by the β anomers of 2-acetamido-2-deoxy sugars, it appeared worthwhile to assume that the amide group adopts a preferred orientation relative to its neighboring atoms and that it might be possible to establish this orientation from a suitable application of the octant rule. Any conclusions depend on the correctness of two additional assumptions[3]: (1) the ring exists in the C-1 conformation (Reeves, 1951) and (2) the preferred amide orientation is essentially the same for GNAc, GalNAc, and their glycopyranosides.

Examination of a Dreiding model of Me β-GNAc reveals that it is possible to form a relatively poor hydrogen bond involving the amide oxygen and the hydroxyl of carbon 3. In this orientation the plane of the amide group very nearly includes the C-3 equatorial position. Substituents on carbon 1 are then in left rear octants, and an axial hydroxyl (α anomer) would give a distinctly more negative contribution to the Cotton effect than an equatorial hydroxyl. Moreover, carbon-4 substituents are in front octants and, if an octant rule is obeyed, glycosides of GalNAc would exhibit greater negative contributions to the Cotton effect than glycosides of GNAc. Both of these results are contrary to what is observed.

An orientation of the amide such that its plane includes also carbon-5 substituents and with the carbonyl *cis* to the axial hydrogen of carbon 2 satisfies all the observed requirements. The plane may be rotated by a few degrees in either a clockwise or counterclockwise direction without upsetting the qualitative conclusion. In this orientation, both axial and equatorial hydroxyls of carbon 4 give a negative contribution, but the equatorial is far more negative. In the equilibrium mixture of GNAc, substituents on carbons 1 and 3 would give cancellation effects by symmetry in the β anomer, whereas in the α anomer the hydroxyl on carbon 1 would add much more positive rotation than the negative contribution by the hydroxyl of carbon 3 with a net positive change in the Cotton effect.

In this preliminary discussion of preferred orientation, we do not exclude the possibility that the amide spends some of its time hydrogen bonded to the hydroxyl of carbon 3. Indeed, substitution at this position by another sugar may result in a negative contribution simply by increasing the probability that the amide is oriented as described above because no hydrogen bond can be formed involving the hydroxyl on carbon 3.

As far as the blood group substances are concerned, a detailed discussion of the results cannot yet be presented because of an unknown background due to the amino acids and because of the much greater diversity of composition and substitution in these substances than, for example, in Type XIV pneumococcal polysaccharide. Certain features do, however, stand out, and these merit brief summary. The pronounced Cotton effect in all the blood group substances is unequivocally due to the 2-acetamido group, and its magnitude implies substantial numbers of β-linked GNAc residues. Values of $[m]_{218} - [m]_{300}$ are uniformly lowest in blood group A, and this is at least compatible with the analytical result that there are fewer α-GalNAc residues in H substance than in A substance and that terminal nonreducing α-GalNAc residues in group A are replaced by α-Gal residues in group B (*cf.* Kabat, 1956; Morgan, 1960; Painter *et al.*, 1965). It should be noted also that blood groups A_2 and H are serologically related (*cf.* Kabat, 1956).

Finally, it may be pointed out that most of the amino acid residues in the blood group substances are clearly not part of α-helical segments. Even if one-fifth of the amino acid residues were in such segments, the ORD spectrum would have exhibited a discernible shoulder at 233 mμ. A smaller fraction, of course, could exist in this ordered conformation and escape detection.

The ORD studies raise certain interesting questions from the immunochemical standpoint. If preferred conformations exist involving the orientation of the plane of the 2-acetamido group with respect to the rest of the sugar ring and if these conformations are altered by substitution of sugar residues on other carbons of the amino sugar, an unappreciated new dimension of immunological specificity for nonordered mucopolysaccharides becomes possible (*cf.* Kabat, 1957) which may contribute substantially to or limit the heterogeneity of the antibody response to a single antigenic determinant. Thus one might have antibody combining sites with specificity for one or another or all conformations of the determinant. Furthermore, the removal or substitution of groups on an antigenic determinant could cause a substantial change in its conformation in solution, thereby altering its capacity to react with antibody. Several observations would be compatible with these assumptions. Thus, McCarty (1964) in studying the glycerol teichoic acid of streptococcus found that certain antisera react only with the teichoic acid containing alanine esterified with the OH of carbon 2, others react only with the teichoic acid lacking alanine, while a third group reacts equally well with polymers containing or lacking alanine. The latter type of antisera would have been formed to a conformation involving

[3] It has been suggested by Schellman and Oriel (1962) that the front octants are relatively unimportant in the case of amides and that, accordingly, rigidly oriented amides follow a quadrant rule. There is insufficient experimental evidence on this point, and the theoretical basis of the octant rule is not yet secure (Urry and Eyring, 1964; Tinoco, 1962). Our analysis, because of its limited nature, does not critically depend on the front octants. A full conformational analysis of 2-acetamido-2-deoxy sugars must, however, await a decision based on further theoretical and experimental work.

the –CH– aspect of carbon 2 of the glycerol teichoic acid and not involving the hydrophilic hydroxyl side of the molecule. The findings in studies of the blood group A–B cross-reacting antibody (Schiffman and Howe, 1965) may be interpreted as indicating that this antibody is formed to a conformation of the A determinants which does not involve the 2-acetamido-2-deoxy region of the terminal nonreducing GalNAc residues. In the B determinant GalNAc is replaced by Gal and thus an antibody combining site not involving the 2-acetamido group of the A determinant should not be able to distinguish between A and B. Further correlations of immunochemical reactivity and conformation measured not only by ORD but also by circular dichroism spectra of oligosaccharide determinants of specificity will be carried out to evaluate these possibilities.

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