Circular Dichroism of Oligosaccharides Containing Neuraminic Acid†

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ABSTRACT: In order to test the usefulness of circular dichroism in stereochemical and structural studies of oligosaccharides of glycoproteins, we measured the circular dichroism (CD) for *N*-acetylneuraminic acid (NAcNA) and several derivatives. By acidic methanolysis, we have prepared the deacetylated methyl ester, methyl glycoside of NAcNA, as well as a saponified product. Circular dichroism of these compounds allows us to assign the transition due to the carboxylate chromophore distinct from that due to the amide chromophore. There is a carboxyl n-π^* transition at about 220 nm which has a negative CD band associated with it for the β-methoxyneuraminic acid, but changes sign for the methyl ester (methyl (methyl β-D-neuraminid)ate). We isolated the trisaccharides *N*-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose [(2→3)NAcN-Lac] as well as (2→6)NAcN-Lac by paper chromatography and compared the CD for each. The two isomers show similar but distinguishable CD patterns, with a weak negative band due to the carboxyl group centered at 225 nm and a stronger positive band at 200 nm containing contributions from both the amide and carboxyl groups.

By the application of circular dichroism (CD) to the study of carbohydrates one can gain information on the stereochemistry and anomeric configuration of intersaccharide linkages, and possibly information on the conformation of oligosaccharides in solution. Since this technique requires sample sizes in the range of 0.1 to 2 mg and the samples are not degraded by the measurement it is applicable to studies of biological compounds. We would expect sialic acids to show several Cotton effects arising from two different chromophores on the molecule.

In the present study, we investigate the Cotton effects of NAcNA[1] and of two isomeric trisaccharides containing NAcNA. This nine carbon atom sugar depicted in Table I has an amide attached to C-5 which is similar to the chromophore of the 2-deoxy-2-amido sugars. In addition, NAcNA has a carboxylate at C-1, which is similar to that of uronic acids but with a slightly different electronic structure. The p*K* of NAcNA is low (2.6) and the compound is susceptible to degradation in acidic media so it can be readily studied only as a carboxylate anion.

Previous studies on the CD of 2-deoxy-2-amido sugars show Cotton effects in the 210- and 190-nm regions (Kabat et al., 1969; Stone, 1971). The Cotton effect at 210 nm is apparently due to the amide n-π^* transition and is thus analogous to the polypeptide α-helix band at 220 nm. The CD of the n-π^* transition in 2-deoxy-2-amido sugars has been treated theoretically by Yeh and Bush (1974). Since 190 nm is the ultraviolet absorption maximum due to the amide π-π^* transition in amido sugars, the CD band at that wavelength may be plausibly assigned to the amide π-π^* transition (Stone, 1971). The protonated form of the carboxyl group of uronic acids (pH 2.5) undergoes a weak n-π^* transition at 220–235 nm and a stronger one at 205 nm (Listowsky et al., 1972).

In oligosaccharides from glycoproteins and glycolipids sialic acid is generally found at the nonreducing terminal position. It is connected by a ketosidic bond at C-2 which is directly adjacent to the carboxylate chromophore at C-1. Thus, we expect the Cotton effects due to this chromophore to be especially sensitive to the details of the intersaccharide linkage.

In order to explore this possibility we measured the CD of two isomeric trisaccharides, (2→3)NAcN-Lac and (2→6)NAcN-Lac shown in Table II which are commonly found in human milk and in bovine colostrum. Also, as an aid in establishing which CD bands of NAcNA are due to the carboxylate and which are due to the amide, we studied the CD of two de-N-acetylated derivatives of NAcNA.

Materials and Methods

Analytical Methods. The concentrations in solution of the two NAcN-Lac isomers and the two de-N-acetylated model compounds were determined by Ehrlich's method in order to calculate molar extinction coefficients and molar ellipticities (Schneir and Rafelson, 1966). This test is sensitive in the range of 5–100 μg of NAcNA. On paper and thin-layer chromatograms the locations of the sugars having free amino groups, i.e. the amino acid β-methoxyneuraminic acid and the ester methyl (methyl β-D-neuraminid)ate, were detected by the cupric nitrate–ninhydrin test (Brenner et al., 1969). This consisted of two solutions which were mixed immediately before use. Solution A contained 10 ml of acetic acid and 2 ml of collidine in a solution of 1.0 g of ninhydrin in 50 ml of absolute ethanol. Solution B contained 0.5 g of cupric nitrate dissolved in 50 ml of absolute alcohol. After the chromatogram was sprayed with a mixture of 50 ml of solution A and 3 ml of solution B it was placed in an oven at 105° for 5 min or until the color developed. These compounds show characteristic colors. The *N*-acetyl groups and the free amino groups of all derivatives of neuraminic acid could be detected by the tolidine–iodide method (Nitecki and Goodman, 1966). The dried chromatograms were sprayed with a fresh 10% solution of commercial Clorox bleach in water and left in a fume hood at

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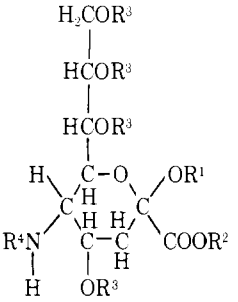
[1] Abbreviations used are: NAcNA, *N*-acetylneuraminic acid; NAcN-Lac, *N*-acetylneuraminyllactose.

Table I:



No.	Name	R^1	R^2	R^3	R^4
I	*N*-Acetylneuraminic acid	H	H	H	CH_3CO
II	Methyl (methyl β-D-neuraminid)ate	CH_3	CH_3	H	H
III	β-Methoxyneuraminic acid	CH_3	H	H	H
IV	Peracetylated derivative of methyl (methyl β-D-neuraminid)ate	CH_3	CH_3	CH_3CO	CH_3CO
V	Peracetylated derivative of β-methoxyneuraminic acid	CH_3	H	CH_3CO	CH_3CO
VI	Pertrimethylsilyl derivative of methyl (methyl β-D-neuraminid)ate	CH_3	CH_3	$(CH_3)_3Si$	H

room temperature for about 30 min and then sprayed with ethanol. They were then dried another 10 min and sprayed with a solution of 5 g of KI and 1 g of *o*-tolidine in 100 ml of acetic acid in 900 ml of water. The deep blue spots fade rather rapidly.

Sample Preparation. Synthetic crystalline *N*-acetylneuraminic acid (95% pure) as well as the mixed isomers of *N*-acetylneuraminyllactose (from bovine colostrum) were obtained from Sigma.

The two trisaccharides were separated by descending chromatography on Whatman No. 3 paper for 20 to 24 hr in pyridine–ethyl acetate–acetic acid–water (5:5:1:3, v/v) (solvent system I). The isomers were detected by spraying with the tolidine–iodide reagent. After chromatography the paper strips were treated to remove the solvent system residues which would interfere with absorption and circular dichroism spectroscopy. Thoroughly dried strips were exposed to an NH_3 atmosphere to break up pyridine–acetic acid salts, and then refluxed in diethyl ether for 15 min and dried again. The samples were eluted from the paper strips into 0.5 ml of water and stored at −10°.

The methyl (methyl β-D-neuraminid)ate (structure II) was obtained by methanolysis of NAcNA (Klenk and Faillard, 1954). This was accomplished by heating 10 mg of NAcNA dissolved in 5 ml of 5% methanolic HCl (5 g of gaseous HCl dissolved in 95 g of reagent grade methanol) for 3 hr at 100–110° in a sealed Pyrex bomb tube. The methanolic HCl was removed after the reaction by evaporation. The product was purified by ascending chromatography on Whatman No. 40 paper for 18 hr in butanol–acetic acid–water (4:1:1, v/v) (solvent system II). The product was detected with the cupric nitrate–ninhydrin spray. The sample was eluted from the paper strip in 5 ml of water and stored at −10°. This synthetic procedure leads to β configuration (Kuhn et al., 1966; Yu and Ledeen, 1969).

Mass spectra of volatile derivatives of methyl (methyl β-D-neuraminid)ate were taken to verify the correct identity of this compound. The trimethylsilyl derivative (structure VI) was synthesized by adding one drop of 1,1,1,3,3,3-hexamethyldisilazane, one drop of chlorotrimethylsilane, and three drops of pyridine to the dried sample in a 0.3-ml conical Reacti-vial. Samples were purified by gas chromatography. The peracetylated derivative of methyl (methyl β-D-neuraminid)ate (structure IV) was synthesized for mass spectroscopy by adding 0.046 ml of acetic anhydride and 0.053 ml of pyridine to the dried sample, and leaving this at room temperature for 18 hr. The reaction was stopped by removal of the pyridine and acetic anhydride.

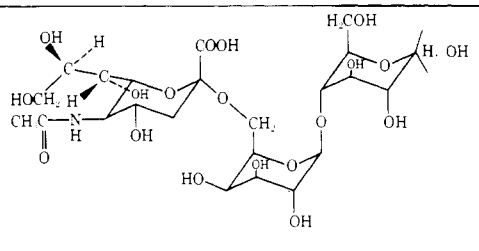
The β-methoxyneuraminic acid (structure III) was obtained by saponification of the methyl ester in aqueous 2 *M* NH_4OH solution at room temperature for 1 hr (Weygand and Rinno, 1957). The dried product was purified for optical spectroscopy by descending paper chromatography for 24 hr in solvent system II. This sample was also eluted in 0.5 ml of water and stored at −10°. The peracetylated derivative (structure V) was made as described for the ester above.

Gas–liquid chromatography was carried out on a Varian Aerograph series 2400 machine equipped with a flame ionization detector. All samples were chromatographed on a glass 6 ft 3% SE-30 column with 80–100 mesh Chromosorb W H/P as solid support.

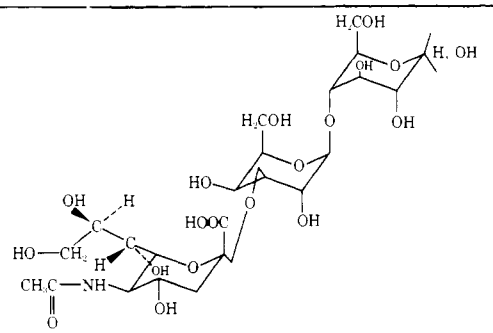
Spectroscopy. The mass spectra of the volatile derivatives of the methyl (methyl β-D-neuraminid)ate and the β-methoxyneuraminic acid were measured on a CH 7 Varian MAT mass spectrometer.

Circular dichroism spectra were measured on a Cary 60 with a 6003 circular dichroism accessory. The data were recorded both on a strip chart and on magnetic tape and smoothed by fast Fourier transform algorithm (Bush,

Table II



VII (2→6)-*N*-Acetylneuraminyllactose



VIII (2→3)-*N*-Acetylneuraminyllactose

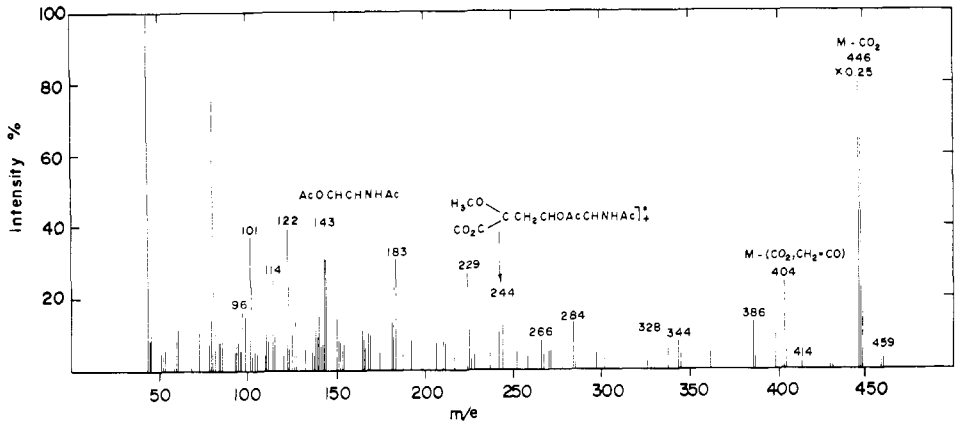


FIGURE 1: Mass spectrum of the peracetylated derivative of β-methoxyneuraminic acid. M stands for molecular ion, *m/e* 490.

1974). The smoothed spectra were decomposed into simple gaussian curves.[2]

Absorption spectra were measured on a Cary 15 instrument flushed with water pumped nitrogen for measurements below 200 nm. The solvent was water for all compounds for both CD and absorbance measurements. The *N*-acetylneuraminic acid solution was buffered with 0.001 *M* Tris at pH 7.05. The pH values of the other acids were between 5 and 8 (carboxylate form).

Results

Chromatography. Descending paper chromatograms in solvent system I of the commercial NAcN-Lac mixture yielded three spots when treated with the tolidine–iodide spray. The NAcN-Lac mixture is specified to be 63% of (2→3)NAcN-Lac and 12% of (2→6)NAcN-Lac. Under these chromatography conditions the relative migration of (2→6)NAcN-Lac with respect to (2→3)NAcN-Lac ($R_{(2\rightarrow3)\text{NAcN-Lac}}$) is 0.75 (Schneir and Rafelson, 1966). We numbered our chromatogram spots 1, 2, and 3, with 1 referring to the fastest migrating spot and 3 the slowest. We found that R_1 for spot 2 is 0.6–0.75, but R_2 for spot 3 is only 0.2–0.4. Although the tolidine–iodide spray produces a darker and larger reaction for spot 2 than for spots 1 and 3, the absorbance spectra, the CD spectra, and Ehrlich's test all indicated that spot 1 was richest in neuraminic acid as is the (2→3)NAcN-Lac in the commercial mixture. Thus, we concluded that spot 1 was (2→3)NAcN-Lac while spot 2 was (2→6)NAcN-Lac in agreement with the chromatographic results of Schneir and Rafelson (1966).

The *N*-acetylneuraminic acid, when chromatographed on silica gel thin-layer plates in ethyl acetate–pyridine–water (2:1:1, v/v) and detected with tolidine–iodide spray for amide, has a major component with R_f 0.26 and a minor contaminant with R_f 0.6. Since the contaminant amounted to only 5% of the total, we measured the CD without further purification.

The crude methyl (methyl β-D-neuraminid)ate shows only one major component with R_f 0.31 in solvent system II on paper when sprayed with either the tolidine–iodide or the cupric nitrate–ninhydrin sprays.

The saponification of the methyl ester (structure II) with 2 *M* NH_4OH resulted in two major products and several minor products under solvent system II. The product which was bright pink after being sprayed with the cupric nitrate–ninhydrin reagent and had R_f 0.25 (silica gel plates, ascending) was shown to be the β-methoxyneuraminic acid by mass spectroscopy.

Our gas–liquid chromatography experiments were carried out primarily to collect pure samples from mass spectroscopy. The retention times were consistent with those given by Sweeley and Walker (1964). We were successful in purifying a sample of the pertrimethylsilyl derivative of methyl (methyl β-D-neuraminid)ate for mass spectroscopy.

Spectroscopy. The mass spectrum obtained from our sample of the pertrimethylsilylated derivative of methyl (methyl β-D-neuraminid)ate purified by gas chromatography is consistent with previous results (Sweeley and Vance, 1967). That of the peracetylated derivative of methyl (methyl β-D-neuraminid)ate agrees with the one of Kochetkov et al. (1973) with the exception that we find a small peak at 474 *m/e* units which corresponds to a loss of the methyl glycosidic OCH_3 group from the parent.

The mass spectrum of the peracetylated derivative of β-methoxyneuraminic acid has not been reported previously and is presented in Figure 1. The fragmentation pattern is similar to that of the methyl ester (structure IV). The masses of fragments which contain the carboxylic acid group are 15 *m/e* units smaller than the corresponding fragments for structure IV. The relative intensity of the peak at *m/e* 446 (structure IV minus $COOCH_3$, or structure V minus CO_2) is much greater for the peracetylated derivative of β-methoxyneuraminic acid than for the ester. The similarities in the fragmentation pathways indicate that the products of saponification have been identified correctly.

The absorption spectra for *N*-acetylneuraminic acid and for methyl (methyl β-D-neuraminid)ate are shown in Figure 2. As one would expect, the absorption spectra of the two trisaccharides (not shown) are essentially the same as for the *N*-acetylneuraminic acid. Due to the electronically forbidden nature of the carboxylate band of the β-methoxyneuraminic acid, the lowest energy absorption band would be the π–π^* of the carboxylate, the maximum of which is at a shorter wavelength than 185 nm (Barnes and Simpson, 1963). The influence of the π–π^* band can be recognized in the CD spectrum of β-methoxyneuraminic acid (see below). The absorption spectrum of *N*-acetylneuraminic acid contains four bands: the two n–π^* and the two π–π^* transitions for the carboxylic acid group and the *N*-acetyl group. As a

[2] We used an iterative nonlinear least-squares fit program adapted for circular dichroism spectra from a program available from J. P. Fackler, Jr., Department of Chemistry, Case Institute of Technology, Cleveland, Ohio 44106.

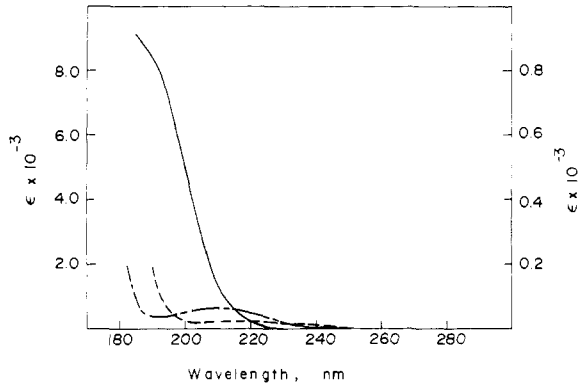


FIGURE 2: Ultraviolet absorption spectra of two neuraminic acid derivatives and ethyl acetate: (—) *N*-acetylneuraminic acid (NAcNA); (- - -) methyl (methyl β-D-neuraminid)ate; (- - - -) ethyl acetate. The scale for ethyl acetate is on the right; the scale for the other two is on the left.

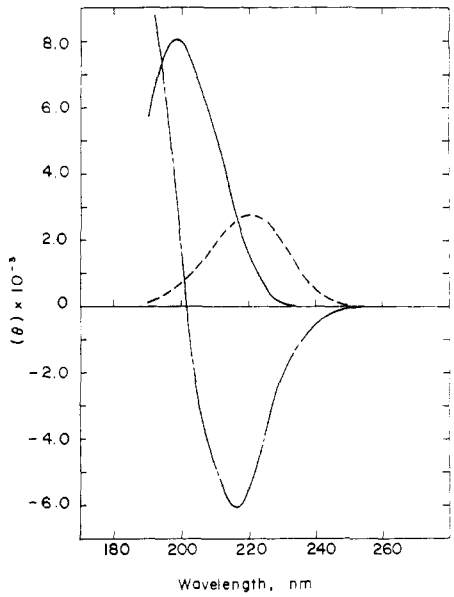


FIGURE 3: Circular dichroism spectra of three neuraminic acid derivatives: (—) *N*-acetylneuraminic acid; (- - -) methyl (methyl β-D-neuraminid)ate; (- - - -) β-methoxyneuraminic acid. These are plotted from fast Fourier transform output.

result of overlap, we cannot distinguish the individual bands in this absorption spectrum.

A weak n–π* transition for the methyl ester group at 215–220 nm is observed in the absorption spectrum of the methyl (methyl β-D-neuraminid)ate (extinction coefficient 150). In order to show that this spectrum is reasonable, we studied the absorption spectrum of ethyl acetate in hexane (shown also in Figure 2) (Barnes and Simpson, 1963). This compound has an n–π* transition at 210 nm with a molar extinction of about 60.

The circular dichroism spectra of five neuraminic acid derivatives are shown in Figures 3 and 4. The spectrum of methyl (methyl β-D-neuramid)ate shows a single positive band centered at 221 nm. On saponification to the acid we obtain a negative band centered at 217 nm and a positive band centered below 190 nm. Neither of the de-N-acetylated compounds contains a contribution due to the amide chromophore. The *N*-acetylneuraminic acid spectrum has a peak at 199 nm with a shoulder at 210 nm. This spectrum contains contributions from both the amide n–π* (210 nm)

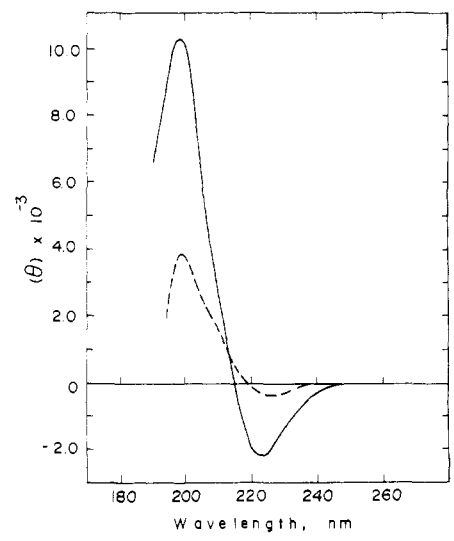


FIGURE 4: Circular dichroism spectra of the two milk trisaccharides *N*-acetylneuraminyl(2→3)lactose (—) and *N*-acetylneuraminyl-(2→6)lactose (- - -) plotted from fast Fourier transform output.

and π–π* (190 nm) as well as from the carboxylate n–π* (217 nm). Since all of these bands are apparently positive, it is not easy to discern them in the spectrum of NAcNA. This spectrum is consistent with previous results (Stone, 1971). The spectra of the two *N*-acetylneuraminyllactose isomers are similar in shape, but differ in magnitude. They both have negative bands near 225 nm which differ slightly in position. The (2→6)NAcN-Lac has a band associated with the carboxylate n–π* at 228.2 nm while the corresponding band for (2→3)NAcN-Lac is at 224.2 nm. The larger positive band for both compounds at 198 nm contains contributions from electronic transitions of the amide.

Decomposition of CD Spectra into Gaussian Bands. Since NAcNA and the trisaccharides have several contributing chromophoric transitions, we have decomposed the CD spectra into gaussian bands to assist in interpretation of the curves. For the gaussian decomposition, we used a digital computer program[2] which fits the observed CD spectrum to a selected number of gaussians by an iterative nonlinear least-squares procedure. The reader should be aware that although they may be helpful in interpreting complex CD curves, gaussian decompositions are not unique. Therefore, our results (Table III) should be viewed with some reservations. A tentative assignment of each CD band to an electronic transition is given along with an approximate rotational strength computed from standard formulas for gaussian bands.

Discussion

In Table III we have indicated tentative assignments of the CD bands to electronic transitions. We may be reasonably certain of our assignment of the carboxylate n–π* transition in the 216–226-nm region. In the de-N-acetylated derivatives of NAcNA, Cotton effects due to the amide chromophore are not present. In the methyl (methyl β-D-neuraminid)ate we see a band at 221 nm with an ellipticity ([θ]) of 2770. We may assign this CD band to the electronically forbidden carboxyl ester n–π* absorption band (Figure 2). In the β-methoxyneuraminic acid we see a negative band, $[\theta] = -5000$, at 217 nm which can be assigned to the carboxylate n–π* transition by analogy with the results of Listowsky et al. (1972) on uronic acids. The carboxylate

Table III: Gaussian Decomposition of CD.

Compound	Electronic Transition	Wavelength (nm)	Peak Height $[\theta] \times 10^{-3}$	Half-Width at Half-Maximum (nm)	Rotational Strength (cgs-esu $\times 10^{-40}$)
Methyl (methyl β-D-neuraminid)ate	Ester, n–π*	221	2.8	13.3	8.30
β-Methoxy-neuraminic acid	Carboxylate, n–π*	217	−5.0	11.3	−12.8
	Carboxylate, π–π*	189	10.1	8.0	21.1
NAcNA	Carboxylate, n–π*	216	0.4	4.7	0.38
	Amide, n–π*	210	0.5	4.0	0.48
	?, π–π*	198	8.1	12.4	24.62
(2→3)NAcN-Lac	Carboxylate, n–π*	220	−3.0	9.0	−6.03
	Amide, n–π*	207	−2.2	4.7	−2.43
	?, π–π*	199	10.9	10.9	29.3
(2→6)NAcN-Lac	Carboxylate, n–π*	226	−0.5	8.1	−0.9
	Amide, n–π*	211	0.8	5.9	1.0
	?, π–π*	199	3.8	7.0	6.6

n–π* CD bands are easily measured with good precision due to the relatively low ultraviolet (uv) absorption associated with the electronically forbidden n–π* transition. The difference in sign of the n–π* CD between the carboxylate and that of the carboxylate ester could arise from the differences in the electronic structure of the two chromophores. Moreover the added steric constraint of the methyl ester inhibits rotation about the C_1–C_2 bond when compared to the essentially free rotation of the small carboxylate group.

In NAcNA the carboxylate is adjacent to a free hydroxyl rather than to a glycosidic bond and the carboxylate n–π* CD band is apparently positive. Therefore, it is not prominent in the CD spectrum of NAcNA due to the presence of the strong positive Cotton effects of the amide chromophore (Figure 4). In contrast, in both the NAcN-Lac trisaccharides, the carboxylate n–π* band has negative CD leading to troughs in the 224–228-nm region. We find a slight difference in the position of the troughs between these two compounds. For an average of 16 spectra of (2→3)NAcN-Lac, the trough falls at 224.2 nm with a standard deviation of 1.8 nm, while the band in (2→6)NAcN-Lac falls at 228.2 nm with a standard deviation of 2.2 nm.

It is somewhat surprising that the carboxylate n–π* CD is similar in the NAcN-Lac isomers which have the α-ketosidic linkage and in our synthetic methyl ketoside which has the β-anomeric conformation (Yu and Ledeen, 1969, 1970). One might have expected the signs of the bands to differ allowing one to use the CD at 220 nm as an analytical tool for determining the anomeric configuration.

The CD of NAcNA and of the two NAcN-Lac isomers in the 190–210-nm region contains contributions both from the amide at C-5 as well as from the carboxylate chromophore. For all these compounds, Table III indicates a CD band at 207 to 211 nm which is similar to that reported for the 2-deoxy-2-amido sugars and it may be assigned to the amide n–π* transition (Yeh and Bush, 1974). Our gaussian decomposition indicates a positive sign for this band in (2→6)NAcN-Lac and a negative sign for the band in the (2→3) isomer. This result is somewhat surprising since the obvious difference between the isomers is located at a distance from the amide chromophore. While it is possible that this result is an artifact of the gaussian decomposition, all of our individual CD curves for (2→6)NAcN-Lac indicate a small positive shoulder at 210 nm which is not seen in the curve for the (2→3) isomer.

The band at 199 nm is somewhat puzzling but it is quite prominent in the spectra of NAcNA and of the two trisaccharides. The amide π–π* band is generally found at 190 nm (Stone, 1971) and the carboxylate has no CD in the 200-nm region as may be seen in our spectrum of β-methoxyneuraminic acid (Figure 3). It is possible that the band observed at 199 nm results from overlap of the amide π–π* band with an undetected band due to the carboxylate. Our failure to detect this band in β-methoxyneuraminic acid could be due to its small size in the model compound or it could be due to its short wavelength. The β-methoxyneuraminic acid shows positive CD at 190 nm which could arise from a strong carboxylate π–π* transition centered at a wavelength shorter than 190 nm.

In our introductory statement, we suggested that we would expect the CD due to the carboxylate at C-1 to be sensitive to the intersaccharide linkage at C-2. However, the CD for (2→3)NAcN-Lac and (2→6)NAcN-Lac do not differ very dramatically. The magnitudes of the CD for the (2→6)NAcN-Lac are lower than those for the (2→3)NAcN-Lac, perhaps reflecting the greater rotational freedom of the (2→6) linkage as shown in Table II. In the (2→6) linkage, NAcNA is connected to a primary alcohol (C-6 of galactose) while in the (2→3) linkage NAcNA is connected to a secondary alcohol at C-3 of galactose. There is a small but significant difference in the position of the minimum of the CD in the 228–224-nm region for the two isomers. It is not clear whether these slight differences in the CD spectrum would be of analytical value in distinguishing the two compounds.

It is interesting to compare our spectra with those reported by Stone and Kolodny (1971) for several gangliosides which contain similar carbohydrate moieties. Their bovine kidney ganglioside BG_{M3} has a (2→3)-*N*-acetylneuraminyllactose which is linked glycosidically to the ceramide. They report a negative CD band at approximately 220 nm with $[\theta] = -5000$ and with another negative band at 199 nm having $[\theta] = -30{,}000$. The transition they observe at longer wavelengths which is associated with the n–π* transition of the carboxylate gives rise to CD similar to the one we observed in the free oligosaccharide (Figure 4). On the other hand their shorter wavelength band is opposite in sign and roughly three times as large. Stone and Kolodny (1971) claim the ceramide contributes no more than 10% to the CD of the ganglioside at wavelengths shorter than 210 nm. The ceramide, however, contains a fatty acid amide bond at C-2 which could be contributing to the CD in the 210–190-

nm region. On the other hand, it is possible that the ganglioside has the oligosaccharide in quite a different conformation from that in which the free oligosaccharide is found. The ganglioside could form micelles in aqueous solution and this could lead to a dramatic conformational change which we see in the CD spectrum. The differences between our data and those of Stone and Kolodny (1971) on bovine G_{M3} indicate that the ceramide has a substantial influence on the conformation of the oligosaccharide (2→3)NAcN-Lac.

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Effect of Selected Anions and Solvents on the Electronic Absorption, Nuclear Magnetic Resonance, and Infrared Spectra of the *N*-Retinylidene-*n*-butylammonium Cation†

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ABSTRACT: The specific counteranion and the solvent have been shown to regulate the electronic excitation energy of the *N*-retinylidene-*n*-butylammonium cation. Halogenated hydrocarbon solvents which can hydrogen bond with the anion shift the λ_{max} to longer wavelengths, whereas the solvent dipole, acting as a bulk effect, shifts the λ_{max} to shorter wavelength. Here solvents which can donate two hydrogens for hydrogen bonding, such as *cis*- and *trans*-1,2-dichloroethylene and *cis*- and *trans*-1,2-dichlorocyclohexane, are used as solvents for the Cl^-, Br^-, and I^- salts. As expected the cis solvents allow longer wavelengths than do the trans solvents. Results of nuclear magnetic resonance spectroscopy are shown to be in agreement with electronic absorption spectroscopy. The C-1$\hat{1}$ proton and the C-13 and C-9 methyl protons show a considerable downfield shift in the salts with respect to the Schiff base. Furthermore the same protons show a continuing downfield shift as the anions are exchanged from Cl^-, Br^- to I^-. This is in agreement with the interpretation of greater positive charge delocalization as the anions are changed in the above manner. The infrared absorptions of the C=N group in the Schiff base and the protonated form are shown to be almost similar. This is rationalized by showing that the force constant can remain constant as the highly related factors bond order, bond distance, and the effective electronegativity are changed in a self-compensating manner.

A general introduction to the problems concerning the bathochromic shift in *N*-retinylidene-*n*-butylammonium salts, i.e., model chromophores of visual pigments, is given in Blatz et al. (1972). It was shown that in certain solvents the λ_{max} of the salt is a function of the counteranion. The excitation energy of the salt increased linearly with the reciprocal of the square of the distance between the centers of cationic and anionic charges. The limiting value of the excitation energy as $1/d^2$ approaches zero is approximately the excitation energy of the retinylic cation and is equivalent to a λ_{max} of 589 nm. Certain solvents such as methanol, etha-

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