# Immunochemical Studies on Blood Groups. XXXVII. The Structures of Difucosyl and Other Oligosaccharides Produced by Alkaline Degradation of Blood Group A, B, and H Substances. Optical Rotatory Dispersion and Circular Dichroism Spectra of These Oligosaccharides*

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ABSTRACT: Treatment of blood group A, B, and H substances with NaOH–$NaBH_4$ yielded three monofucosyl and three difucosyl oligosaccharides. The structure of the monofucosyl H active oligosaccharide is α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-R (R is $CH_2OHCHOHCH{=}CHCHOHCH_2OH$). The corresponding A and B oligosaccharides have 2-acetamido-2-deoxy-D-galactopyranose and -D-galactose, respectively, linked α-(1→3) to the galactose residue of this structure. The difucosyl oligosaccharides have basically the same structures but with a second fucose which is linked to the *N*-acetylglucosamine residue of the corresponding monofucosyl structures. Methylation of these oligosaccharides and identification of the glucosamine methyl ether formed as 6-*O*-methyl-D-glucosamine show that the fucose is substituted on the C-3 position of the *N*-acetylglucosamine. The optical rotatory dispersion (ORD) spectra of the monofucosyl oligosaccharides show pronounced Cotton effect troughs at about 220 mμ due to the 2-acetamido group of the *N*-acetylhexosamine residues. The circular dichroism (CD) spectra have strong ellipticity bands which are due to the same chromophore. For each pair of oligosaccharides, the difucosyl compound showed considerably larger Cotton effects and ellipticity bands than the monofucosyl oligosaccharides. A related pair of milk oligosaccharides, lacto-*N*-difucohexaose I and lacto-*N*-fucopentaose I, also show this effect. The ORD and CD data thus support the methylation studies on the structure of the difucosyl oligosaccharides. The immunochemical significance of the ORD and CD results is discussed in terms of possible conformations of the blood group active determinants.

The difucosyl oligosaccharides have important implications for the activity and biosynthesis of blood group substances.

Blood group active oligosaccharides having two fucose residues have recently been isolated from A, B, and H blood group substances (Lloyd *et al.*, 1966a). The main chains of these oligosaccharides, on which L-fucose residues occur as branch points, have the following structures

α-D-GalNAc[1]-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-R
A

α-D-Gal-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-R
B

β-D-Gal-(1→4)-β-D-GNAc-R
H

R is 3-hexene-1,2,5,6-tetrol (Lloyd *et al.*, 1966a; Lloyd and Kabat, 1967) and is formed from a galactose residue during the alkaline degradation. One fucose is linked α-(1→2) to the galactosyl residue of these structures as in the corresponding monofucosyl oligosaccharides (Lloyd and Kabat, 1964; Lloyd *et al.*, 1966a; *cf.* Rege *et al.*, 1964; Painter *et al.*, 1965). The second fucose was shown to be substituted on the adjacent *N*-acetylglucosamine residue, although the position of substitution was not determined. The identification of 6-*O*-methylglucosamine following methylation and hydrolysis of the B and H oligosaccharides and its tentative identification from methylated A hexasaccharide has now shown that this fucose is linked to the C-3 position of the *N*-acetylglucosamine.

Optical rotatory dispersion (ORD) spectra have recently been shown to give valuable information about the structure of *N*-acetylhexosamine-containing

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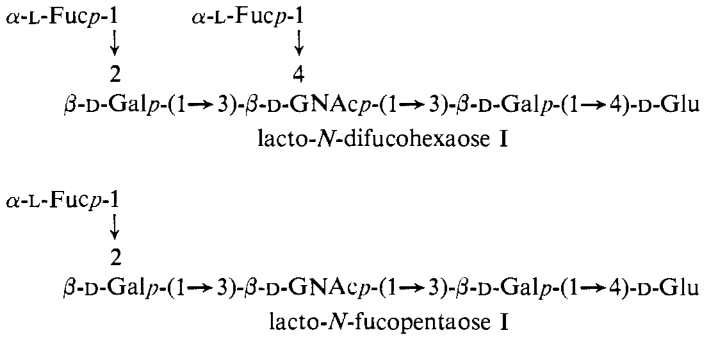
[1] Abbreviations used: GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GNAc, 2-acetamido-2-deoxy-D-glucopyranose; Gal, D-galactopyranose; Fuc, L-fucopyranose; Gluc, D-glucopyranose; TMS, trimethylsilyl; DMF, *N*,*N*-dimethylformamide.



$$\overset{\substack{\alpha\text{-L-Fuc}p\text{-}1\\ \downarrow\\ 2}}{\beta\text{-D-Gal}p}\text{-}(1\rightarrow 3)\text{-}\overset{\substack{\alpha\text{-L-Fuc}p\text{-}1\\ \downarrow\\ 4}}{\beta\text{-D-GNAc}p}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-D-Glu}$$

lacto-*N*-difucohexaose I

$$\overset{\substack{\alpha\text{-L-Fuc}p\text{-}1\\ \downarrow\\ 2}}{\beta\text{-D-Gal}p}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-GNAc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-D-Glu}$$

lacto-*N*-fucopentaose I

FIGURE 1: Structures of milk oligosaccharides.

oligosaccharides (Beychok and Kabat, 1965). Application of this technique and also of circular dichroism (CD) measurements, to the oligosaccharides isolated by alkaline degradation of blood group substances has given additional insight into the structure of these oligosaccharides. Comparison of the spectra with those of the milk oligosaccharides (Beychok and Kabat, 1965) confirms that the second fucose is linked to *N*-acetylglucosamine in the difucosyl oligosaccharides.

An important feature of the ORD spectra is the trough of a negative Cotton effect at about 220 m$\mu$ owing to the 2-acetamido group. Because the intensity and position of this Cotton effect reflect specific features of the orientation of the acetamido group, it is important to separate this contribution to the rotatory dispersion from those arising from other centers of optical activity. Beychok and Kabat (1965) adopted the approximate procedure for this purpose, of subtracting the rotation at 300 m$\mu$ from the rotation at the trough of the Cotton effect. That procedure is used in the present work also. However, a considerably more precise and sensitive means for isolating the optical activity of a chromophore (such as the 2-acetamido group) from the background is by measurement of CD spectra.

In CD spectra, particular chromophores generate discrete bands of fairly narrow half-width, much as in ordinary absorption spectra, except that the bands may be positive or negative in sign. Each of these corresponds to a Cotton effect in the ORD spectrum. It will be recalled that the sign of the Cotton effect is negative if the long-wavelength extremum is negative. The corresponding CD spectrum would exhibit a negative ellipticity band, whose center occurs at the same wavelength as the inflection point, *i.e.*, wavelength of zero rotation of the Cotton effect (for recent review article, see Beychok, 1966). The wavelength of maximum ellipticity thus occurs at a shorter wavelength than the trough of a negative Cotton effect or the peak of a positive Cotton effect. Most importantly, the ellipticity band generated by the acetamido group is not overlapped by the shorter wavelength bands common to all sugars, whereas the acetamido Cotton effect is set upon an uncertain background provided by all these quite intense bands.

## Materials

5-*O*-Methylarabinose was provided by Dr. E. Bishop. 2-*O*-Methyl-D-arabinose was prepared from mustard seed araban (provided by Dr. N. G. Richardson) which gives arabinose, 2-*O*-methyl-, 2,3-di-*O*-methyl-, and 2,3,5-tri-*O*-methylarabinose on methylation and hydrolysis (Hirst *et al.*, 1965). Methylation of 150 mg of araban and separation of the components by paper chromatography yielded 8 mg of 2-*O*-methylarabinose. The blood group active oligosaccharides used in the study were those described earlier (Lloyd *et al.*, 1966a). The milk oligosaccharides were gifts from Professor R. Kuhn; they have the structures shown in Figure 1.

## Methods

Methylation of oligosaccharides was carried out with (a) methyl iodide and $Ag_2O$ in DMF and then (b) methyl iodide and BaO in DMF as described by Lloyd *et al.* (1966a). Gas chromatography was performed on an F & M 810 chromatograph. 2-*O*-Methylarabinose and 5-*O*-methylarabinose were separated as the TMS derivatives of their methyl glycosides using a butane-1,4-diol succinate polyester on Celite (15%) column by elution with nitrogen (30 ml/min) at 150°. The relative elution times are given in Table II. Products of periodate oxidation were examined on a 13% Versamid on Diatoport column at 138° and 40 cc of $N_2$/min (*cf.* Dutton and Unrau, 1965).

ORD measurements were carried out on a Bendix-Ericssen automatic recording spectropolarimeter, Model Polarmatic 62, as described previously (Beychok and Kabat, 1965). CD spectra were measured on a Jouan dichrographe modified for tenfold-increased sensitivity (Beychok, 1965). Equations for calculating molecular ellipticity are given by Moscowitz (1960).

## Experiments and Results

*Isolation of O-Methylglucosamine from Hydrolysates of Methylated Difucosyl Oligosaccharides.* Methylated oligosaccharides ($AR_{IM5}1.0$, $BR_{IM5}1.2$, and $HR_{IM5}2.5$, *ca.* 2 mg each) were hydrolyzed in 2 N $H_2SO_4$ at 100°

for 2–3 hr to give the constituent methyl sugars. The glucosamine methyl ether was then separated from the other sugars in the hydrolysate. In the case of $AR_{IM5}1.0$ this was done by preparative thin layer chromatography (tlc) (Lloyd *et al.*, 1966a). This method was useful in that, in addition to separating the glucosamine methyl ether from the neutral sugars, it also separated it from 3,4,6-tri-*O*-methylgalactosamine. However the yield was rather low and the glucosamine methyl ethers from $BR_{IM5}1.2$ and $HR_{IM5}2.5$ (where glucosamine is the only amino sugar present) were separated from the neutral methyl sugars by ion-exchange chromatography. The mixture of sugars was passed through a column (0.8 × 0.2 cm) of Dowex 50-X12, 100–200 mesh ($H^+$), and the neutral sugars were eluted with water (10 ml). The eluates were evaporated to dryness and the neutral sugars were converted to their methyl glycosides for examination by gas–liquid partition chromatography (glpc) (see Table I). The column was then eluted with 5 ml of 1 N HCl to remove the glucosamine methyl ether. Evaporation of the eluate *in vacuo* over NaOH gave the sugar as its hydrochloride. The product was examined by tlc (on silica gel G, by elution with benzene–methanol, 90:10) to show that it did not contain neutral sugars.

TABLE I: Identification of Methyl Ethers of Neutral Sugars from Methylated Difucosyl Oligosaccharides by Gas Chromatography.

| Oligosaccharide | Methyl Ethers of Neutral Sugars[a] |
|---|---|
| $BR_{IM5}1.2$ | 2,3,4-$Me_3$Fuc (0.50), 2,3,4,6-$Me_4$-Gal(1.14) and 4,6-$Me_2$Gal (1.91 s and 2.60 m)[b] |
| $HR_{IM5}2.5$ | 2,3,4-$Me_3$Fuc (0.50), 3,4,6-$Me_3$Gal (1.58 s and 2.08 m) |

[a] Identified as methyl glycosides on polyphenyl ether column as described previously (Lloyd *et al.*, 1966a). The elution times of each component relative to methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside are given in parentheses. [b] s = strong; m = medium peak.

*Identification of the Hexosamine Methyl Ethers as 6-O-Methylglucosamine.* The glucosamine methyl ether from the column was divided into two portions and the position of the *O*-methyl group was determined by two methods. (a) The sample (in 50 μl of water) was treated with 20 μl of ninhydrin solution (100 mg of ninhydrin in 5 ml of 10% pyridine in water; *cf.* Stoffyn and Jeanloz, 1954). The mixture was heated at 100° for 30 min and then cooled. Any precipitate which formed was centrifuged. The solution was evaporated to dryness *in vacuo* over $P_2O_5$ and the methyl glycosides were formed by heating with 250 μl of 2.5% methanolic HCl at 100° for 3–4 hr. The syrupy material was converted to its TMS derivative by adding 15 μl of reagent (200 μl of hexamethyldisilazane and 100 μl of trimethylchlorosilane in 1 ml of pyridine as described by Sweeley *et al.*, 1963). The TMS methyl glycosides were examined by glpc. The elution times of the glycosides as compared to methyl *O*-(TMS)-α-D-glucoside are given in Table II. The arabinose derivatives derived from $BR_{IM5}1.2$ and $HR_{IM5}2.5$ were both identified as 5-*O*-methylarabinose and, therefore, the methylglucosamine, from which they were formed, is 6-*O*-methylglucosamine. Oligosaccharide $AR_{IM5}1.0$ was not available in a sufficient amount for this technique to be used. (b) The sample was treated with 0.25 mg of $NaBH_4$ in 20 μl of water. On the following day the solution was heated at 100° for 2–3 min, cooled, and 100 μl of 0.1 M $NaIO_4$ solution was added. After 24 hr in the dark at 4°, 2.0 mg of $BaCl_2 \cdot 2H_2O$ in 20 μl of water was added. The precipitate was centrifuged and *ca.* 0.2 mg of $NaBH_4$ was added to the solution. The solution was carefully evaporated to about 50 μl and examined by glpc on a Versamid column at 138°. The *O*-methylglucosamines from $BR_{IM5}1.2$ and $HR_{IM5}2.5$ were studied by this technique; 6-*O*-methyl-D-galactose and 3-*O*-methyl-D-glucose were also examined as controls. The *O*-methyl ethers from the blood group oligosaccharides and also 6-*O*-methyl-D-galactose all gave a peak corresponding to ethylene glycol monomethyl ether (2.5 min), whereas 3-*O*-methylglucose did not. The last compound gave a peak eluted in 22 min (probably 2-*O*-methylglycerol); the other compounds also gave minor peaks in this position. The glucosamine methyl ether from $AR_{IM5}1.0$ was oxidized by periodate as above, except that the first reduction with sodium borohydride was omitted; it also gave a peak at 2.5 min corresponding to authentic ethylene glycol monomethyl ether.

TABLE II: Gas Chromatography of the Arabinose Methyl Ethers Formed by the Ninhydrin Treatment of the *O*-Methylglucosamines from Methylated Difucosyl Oligosaccharides.

| Sample | Rel Retention Times[a] |
|---|---|
| *O*-Methylarabinose from $BR_{IM5}1.2$ | 0.43 (s), 0.51 (m), and 0.70 (trace) |
| *O*-Methylarabinose from $HR_{IM5}2.5$ | 0.43 (s) and 0.51 (m) |
| 5-*O*-Methylarabinose | 0.43 (s) and 0.51 (m) |
| 2-*O*-Methylarabinose | 0.37 (s) and 0.52 (m) |

[a] Times of methyl *O*-(TMS)-glycosides relative to methyl *O*-(TMS)-α-D-glucopyranoside on BDS column at 150°; they are reproducible to within ±0.01 unit.

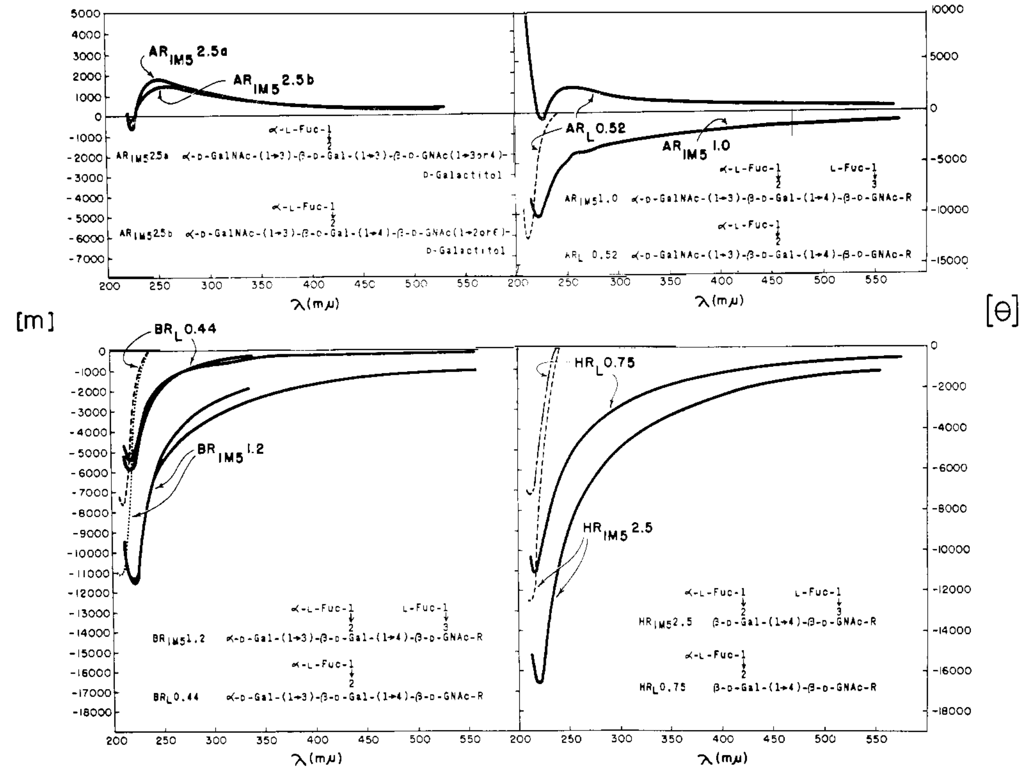


FIGURE 2: Optical rotatory dispersion and circular dichroism curves of A, B, and H oligosaccharides. Solid lines, ORD; broken lines, CD spectra.

TABLE III: ORD and CD Characteristics of Some Fucose-Containing Oligosaccharides.

| Oligosaccharide | ORD<br>$[m]_{\text{trough}} - [m]_{300}$ | CD<br>$[\theta]_{\max}$ |
|---|---|---|
| A hexasaccharide (AR$_{IM5}$1.0) | −5,120 − (−1,500) = −3,620 | — |
| A pentasaccharide (AR$_L$0.52) | 300 − 800 = −500 | −12,200 |
| | Δ = −3,120 | |
| B hexasaccharide (BR$_{IM5}$1.2) | −11,400 − (−3,000) = −8,400 | −11,100 |
| B pentasaccharide (BR$_L$0.44) | −5,700 − (−650) = −5,050 | −7,650 |
| | Δ = −3,350 | Δ = −3,450 |
| H pentasaccharide (HR$_{IM5}$2.5) | −16,600 − (−5,000) = −11,600 | −12,500 |
| H tetrasaccharide (HR$_L$0.75) | −11,150 − (−2,900) = −8,250 | −7,250 |
| | Δ = −3,350 | Δ = −5,250 |
| Lacto-*N*-difucohexaose I | −14,425 − (−2,930) = −11,495[a] | −24,200 |
| Lacto-*N*-fucopentaose I | −7,740 − (−520) = −7,220[a] | −19,200 |
| | Δ = −4,275 | Δ = −5,000 |

[a] These values differ from those given by Beychok and Kabat (1965) since they used $[m]_{218}$ rather than $[m]_{\text{trough}}$ values; the various oligosaccharides give troughs at slightly different wavelengths.

*ORD and CD spectra* for the difucosyl and the corresponding monofucosyl oligosaccharides are shown in Figure 2. Oligosaccharide AR$_{IM5}$1.0 was not available when CD spectra were being taken. Also shown are the spectra of two A oligosaccharides (AR$_{IM5}$2.5a and AR$_{IM5}$2.5b) which resemble the AR$_L$0.52 pentasaccharide but are terminated at the reduced end by galactitol rather than 3-hexene-1,2,5,6-tetrol. Table

III shows the values for $[m]_{\text{trough}} - [m]_{300}$ for the ORD spectra and $[\theta]_{\text{max}}$ values for the CD spectra of the various oligosaccharides. Values for the milk oligosaccharides, lacto-*N*-difucohexaose I and lacto-*N*-fucopentaose I, are also given for comparison.

## Discussion

The isolation of difucosyl oligosaccharides from blood group A, B, and H substances (Lloyd *et al.*, 1966a) was particularly interesting since they had relatively low blood group inhibitory activities as compared with the corresponding monofucosyl oligosaccharides (Lloyd *et al.*, 1966b). Structural studies involving analysis, partial acid hydrolysis, periodate oxidation, and methylation showed that three of these oligosaccharides, one from each substance, had structures similar to the corresponding monofucosyl oligosaccharides but with a second fucose residue attached to an *N*-acetyl-D-glucosamine residue in the oligosaccharide chain. Since no hexosamine methyl ethers were identified during methylation studies, the assignment of the position of the second fucose residue was made only on the evidence that it was not attached to any of the other sugars in the oligosaccharide.

In this study the glucosamine methyl ethers formed by methylation and hydrolysis of the A, B, and H oligosaccharides were examined. The identification of the *O*-methylhexosamine from the B and H oligosaccharides was simplified since glucosamine is the only hexosamine present in these compounds. In these cases the neutral sugars were first removed by absorbing the hexosamine derivatives on Dowex 50 resin. They were identified by gas chromatography of their methyl glycosides (Table I) and were identical with the results reported earlier (Lloyd *et al.*, 1966a); this shows that complete methylation of the oligosaccharides was obtained. The hexosamines were then isolated by eluting the resin with 1 N hydrochloric acid. This separation ensured that any fragments later identified were derived from the hexosamine and not from any of the other constituent sugars. After methylation and hydrolysis of the A oligosaccharide 3,4,6-tri-*O*-methylgalactosamine, which is derived from the terminal nonreducing end of the molecule, was separated from the glucosamine derivative by tlc (Lloyd *et al.*, 1966a).

Since authentic samples of glucosamine methyl ethers were not available to us, the identification of the methyl sugars was carried out using degradative techniques. In the first method the glucosamine methyl ether was degraded by ninhydrin to the corresponding arabinose methyl ether. This is a technique which was first used by Gardell *et al.* (1950) and later modified by Stoffyn and Jeanloz (1954) to distinguish between glucosamine and galactosamine. The *O*-methylarabinose formed was converted to its methyl glycoside and examined by glpc. The elution times of the products were indicative of monomethyl arabinosides rather than tri- or dimethyl arabinosides which have much faster elution times under the conditions used. However, the sugar could not be identified definitively because of variable elution times and the presence in this portion of the chromatogram of other peaks presumably owing to by-products of the ninhydrin degradation. Conversion of the methyl arabinosides to their TMS derivatives using the method of Sweeley *et al.* (1963) gave definitive chromatograms by which the arabinose methyl ether could be identified. Since a β-Gal-(1→4)-GNAc linkage has been identified in these oligosaccharides by partial acid hydrolysis, the fucose can only be substituted on either C-3 or C-6 of the glucosamine residue (assuming only pyranose sugars). Therefore, the only two possible glucosamine monomethyl ethers are 6-*O*-methyl- or 3-*O*-methylglucosamine and the corresponding arabinose sugars, 5-*O*-methyl- and 2-*O*-methylarabinose. The methylglucosamine from the two oligosaccharides studied by this method ($BR_{IM5}1.2$ and $HR_{IM5}2.5$) gave rise to 5-*O*-methylarabinose (see Table II) rather than the 2-*O*-methyl isomer and is, therefore, 6-*O*-methylglucosamine. In the second method the glucosamine methyl ether was first reduced to the alcohol with sodium borohydride, and then completely oxidized with sodium periodate. The products were reduced with sodium borohydride and examined by gas chromatography on a Versamid column (Dutton and Unrau, 1965). The glucosamine ethers from $HR_{IM5}2.5$ and $BR_{IM5}1.2$ both gave a peak corresponding to ethylene glycol monomethyl ether. 6-*O*-Methyl-D-galactose when treated under identical conditions also gave this peak whereas 3-*O*-methyl-D-glucose did not. This shows that the glucosamine is *O*-methylated on its primary hydroxyl group. The glucosamine methyl ether isolated from $AR_{IM5}1.0$ was oxidized with periodate and the product was reduced with sodium borohydride with the expectation of detecting a glycerol methyl ether (Dutton and Unrau, 1965); although no peak was observed corresponding to 1-*O*-methylglycerol, again a peak corresponding to ethylene glycol monomethyl ether was obtained. This indicates that the glucosamine methyl ether from $AR_{IM5}1.0$ is also 6-*O*-methylglucosamine. Oxidation and reduction of the *O*-methylglucosamines from the B and H oligosaccharides without prior reduction to the alcohols also gave peaks for ethylene glycol monomethyl ether rather than for *O*-methylglycerol. Presumably in all three sugars, the intermediate *O*-formyl ester had been hydrolyzed, with further oxidation to the two-carbon fragment then occurring (*cf.* Dutton and Unrau, 1965).

The identification of the glucosamine methyl ether formed by methylation of the difucosyl oligosaccharides as 6-*O*-methylglucosamine[2] shows that the second fucose is linked to the C-3 of the internal *N*-acetyl-D-glucosamine residue of each oligosaccharide as shown

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[2] Rao *et al.* (1966) have recently shown that methylation with MeI and $Ag_2O$ in DMF can lead to *N*-methylation of *N*-acetylhexosamines. This was not found in the present study although some of the minor peaks observed in glpc of the methylarabinose derivatives might be due to this effect.

in Figure 2. The presence in these structures of a disubstituted *N*-acetylglucosamine residue is also supported by the ORD and CD spectra of the oligosaccharides. It should be noted that all the unsaturated oligosaccharides which have been examined are mixtures of oligosaccharides terminated by both the D-*threo* and the *erythro* forms of the unsaturated alcohol, R (Lloyd and Kabat, 1967); it is probable that the difucosyl oligosaccharides are also mixtures of these two isomers.

*ORD and CD Studies.* During a study of the ORD spectra of immunochemically reactive carbohydrates, Beychok and Kabat (1965) found that many sugars and oligosaccharides with 2-acetamido-2-deoxy groups exhibited pronounced negative Cotton effects near 220 m$\mu$. This effect is probably an $n$–$\pi^*$ transition of the amide group and its presence suggests that the amide group adopts a preferred orientation relative to the neighboring ring atoms. The intensity of this Cotton effect could in some cases be correlated with the structure of the sugar or oligosaccharides. A finding particularly pertinent to this study was that the milk oligosaccharides in which the internal *N*-acetylglucosamine, in addition to being substituted on C-3 by the adjacent residue in the chain is also substituted on C-4 by a fucose residue, exhibited a large intensification of their trough values over the oligosaccharides in which the *N*-acetylglucosamine is only singly substituted. As shown in Figure 2 the difucosyl and monofucosyl oligosaccharides from blood group substances show comparable differences in the magnitude of the Cotton effects. For each pair of oligosaccharides, the negative trough of the Cotton effect of the difucosyl oligosaccharides is much greater than the trough given by the monofucosyl compounds. Although the magnitude of the molar rotations of the A, B, and H oligosaccharides differ quite considerably, the difference between the $[m]_{\text{trough}} - [m]_{300}$ values for the three pairs of oligosaccharides is relatively constant and approximately the same as that found for the corresponding milk oligosaccharides (Table III). Thus the differences ($\Delta$) between $[m]_{\text{trough}} - [m]_{300}$ values for A, B, and H difucosyl oligosaccharides and those for the monofucosyl oligosaccharides are almost identical at $-3120$, $-3350$, and $-3350$, respectively. This provides additional evidence for the validity of the $[m]_{300}$ subtraction as a way of estimating the contribution of the other optically active centers and eliminating them from the Cotton effect. The correspondence also gives further support to the methylation studies described above. The shape of the spectra of the B and H oligosaccharides resemble those of the milk oligosaccharides. Indeed the structure of the two H oligosaccharides resembles closely the structures of lacto-*N*-fucopentaose I and lacto-*N*-difucohexaose I except at the reducing ends; the blood group oligosaccharides have an unsaturated alcohol at the reduced end whereas the milk oligosaccharides are terminated with a lactose residue. Another important difference is that the blood group oligosaccharides have a $\beta$-Gal-(1→4)-GNAc linkage in the oligosaccharide chain but in the milk oligosaccharides the linkage is $\beta$-(1→3) and the fucose residue is correspondingly linked to the C-4 of the *N*-acetylglucosamine rather than to C-3 as in the blood group oligosaccharides. For the pair of milk oligosaccharides the $\Delta[m]_{\text{trough}} - [m]_{300}$ value is somewhat higher ($-4455$) than the $\Delta$'s obtained for the blood group oligosaccharides.

The spectra of the A oligosaccharides differ from those of the milk and the B and H oligosaccharides in shape. This effect is particularly noticeable in the spectra of the monofucosyl A oligosaccharides. In these spectra the negative Cotton effects of the *N*-acetylgalactosamine and *N*-acetylglucosamine residues are set upon a strong positive background and the sum of these yields a region of well-discerned anomalous dispersion. A further use of ORD as a structural tool can be made with reference to oligosaccharides $\text{AR}_{\text{IM5}}2.5\text{a}$ and $\text{AR}_{\text{IM5}}2.5\text{b}$. The spectra of these two compounds closely resemble the spectrum of $\text{AR}_{\text{L}}0.52$ and have an initial positive rotation due to the $\alpha$-GalNAc residue which is then followed by a negative trough at 222 m$\mu$. The negative Cotton effect and the shape of the dispersion curve show that the GNAc residue in both oligosaccharides is $\beta$ linked; this point had not previously been established.

The CD spectra of the oligosaccharides (Figure 2) show narrow ellipticity bands and the wavelength of maximum ellipticity occurs at shorter wavelengths than the trough of the negative Cotton effect for each compound. The wavelength of maximum ellipticity is found in the 210–230-m$\mu$ region thus confirming the original identification by Beychok and Kabat (1965) of the 2-acetamido group as the optically active chromophore (*cf.* Beychok, 1966). Since the ellipticity band given by the 2-acetamido group is not strongly overlapped by the bands due to other groups in the sugars, a background correction such as was made for the ORD spectra is not necessary. Table III gives the $[\theta]_{\text{max}}$ values for three pairs of monofucosyl and difucosyl oligosaccharides. In each case the size of the negative band given by the difucosyl oligosaccharides is greater than that given by the corresponding monofucosyl compound. Moreover in two pairs of oligosaccharides, the two H and two milk oligosaccharides, $\Delta[\theta]_{\text{max}}$ values for the di- and monofucosyl compounds are quite close at $-5200$ and $-5000$, respectively. With both the B and the milk oligosaccharides the values for $\Delta[m]_{\text{trough}} - [m]_{300}$ and $\Delta[\theta]_{\text{max}}$ are the same within experimental error. However, with the H oligosaccharides the $\Delta[m]_{\text{trough}} - [m]_{300}$ is $-3350$ while the $\Delta[\theta]_{\text{max}}$ is $-5250$. The significance of this difference cannot at present be explained. The only A oligosaccharide studied has a high $[\theta]_{\text{max}}$ value. It is known that the 2-acetamido Cotton effect exhibited by the $\alpha$-glycoside of *N*-acetyl-D-galactosamine is weak while the ellipticity band is substantial (E. A. Kabat and S. Beychok, paper in preparation). Thus the $[\theta]_{\text{max}}$ for the A pentasaccharide of $-12{,}200$ reflects the additive contribution of both hexosamines and corresponds in magnitude to that of the fucosyl substitution on the hexosamine.

*The Significance of Difucosyl Oligosaccharides for Blood Group Structure and Activity.* The low activities of difucosyl oligosaccharides as compared with the corresponding monofucosyl oligosaccharides in the inhibition of hemagglutination and precipitation has already been described (Lloyd *et al.*, 1966b). The inactivity in the A and B systems of fucose-containing oligosaccharides which lack the terminal, immunodominant *N*-acetyl-D-galactosamine or D-galactose residues, suggests that L-fucose residues are not directly involved in binding to the antibody combining site. Beychok and Kabat (1965), on the basis of ORD studies, have proposed that since the planar amide group of *N*-acetyl sugars assumes a preferred conformation with respect to the sugar ring, oligosaccharide determinants in these polysaccharides also may exist in certain preferred conformations in solution. This suggestion is further confirmed by the study of the CD ellipticity bands associated with the 2-acetamido group of blood group oligosaccharides and other biologically active saccharides (E. A. Kabat and S. Beychok, paper in preparation; *cf.* Beychok, 1966). On this basis it has been suggested (Lloyd *et al.*, 1966b) that the function of the fucose residues in blood group active oligosaccharides is to hold the oligosaccharide chains in a conformation such that the portion of the chain away from the fucose residues is in its optimum conformation for reaction with the antibody combining sites. Examination of models of the A and B determinants will show that many different conformations are possible but that the substitution of an L-fucose on the subterminal galactose residue restricts the number of possibilities. The role of the second fucose in difucosyl oligosaccharides can be viewed as either preventing access of the determinant to the antibody combining site or else in causing another change in the conformation of the main chain such that the determinant no longer reacts as effectively with the antibody combining site. The A, B, and H monofucosyl oligosaccharides have high activity and must represent important antigenic determinants of the blood group substances. However, the significance of this second, less active set of determinants with two fucose residues, is not yet clear. Perhaps when the over-all structure of these substances is elucidated the role of the second fucose in these determinants will become clear.

The presence in blood group substances of determinants having fucose residues attached to the *N*-acetylglucosamine of type 2 ($\beta$-D-Gal-(1→4)-$\beta$-D-GNAc) chains greatly complicates any pathways which might be proposed for the biosynthesis of blood group substances. The oligosaccharides with the second fucose linked (1→3) to the GNAc have been shown not to possess Le$^{a}$ or Le$^{b}$ activity (Lloyd *et al.*, 1966b). Watkins (1966) has recently proposed a scheme for the biosynthesis of soluble blood group substances (*cf.* Watkins and Morgan, 1959) in which it is postulated that the Le gene controls the transfer of L-fucose to the C-4 of *N*-acetylglucosamine in type 1 ($\beta$-D-Gal-(1→3)-$\beta$-D-GNAc) chains. Since in type 2 chains the C-4 position on the glucosamine is already substituted, it is assumed that the Le gene does not act on these chains. If this is the function of the Lewis gene then another gene, not accounted for in the proposed scheme, must be responsible for the fucosylation of the type 2 chains. It is possible that the Lewis L-fucose-*N*-acetylglucosamine transferase has a wide specificity and will transfer to both determinants. The Lewis gene would then be controlling the formation of non-Lewis determinants.

## Added in Proof

Recently Englehard *et al.* (1966) have shown that ORD spectra for oligosaccharides may be considered as being the sum of the contributions of the constituent monomeric residues. On this basis, since methyl $\alpha$-L-fucopyranoside has a high negative rotation ($[m]_{218} - [m]_{300} = -2400$), a substantial portion of the difference between $[m]_{\text{trough}} - [m]_{300}$ values for the difucosyl and monofucosyl oligosaccharides arises from substitution of an additional $\alpha$-L-fucopyranosyl residue to the molecules. The remaining difference reflects the effect of disubstitution on the 2-acetamido Cotton effect; this effect is more clearly illustrated by the CD spectra.

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