Immunochemical Studies on Blood Groups. XXXV. The Activity of Fucose-Containing Oligosaccharides Isolated from Blood Group A, B, and H Substances by Alkaline Degradation*

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ABSTRACT: The study of the blood group activity of some fucose-containing oligosaccharides, isolated from A, B, and H substances by alkaline hydrolysis, has given some insight into the role which L-fucose plays in activity and specificity. The activities of a reduced tetrasaccharide from H substance, which has the structure: α-L-fucosyl-(1→2)-β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucosyl-R (where R is a reduced unsaturated residue) and a reduced pentasaccharide with the same basic structure but with a second fucose, linked to the *N*-acetylglucosamine residue, were studied with a number of anti-H reagents. The tetrasaccharide was active in the inhibition of human, rabbit, eel, and *Ulex* anti-H but the difucosyloligosaccharide was considerably less active. There is some difference in the specificity of the various reagents. The corresponding pairs of oligosaccharides from A and B substances have structures based on these two oligosaccharides but with terminal nonreducing *N*-acetyl-D-galactosamine and D-galactose, respectively, linked α-(1→3) to the galactose residue. The A pentasaccharide, with the fucose linked α-(1→2) to the galactosyl residue, has a much greater capacity to inhibit hemagglutination and precipitation than 2-acetamido-2-deoxy-α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucose but again the difucosyloligosaccharide is much less active. The same pattern of activity was found with the corresponding oligosaccharides from B substance. Although the intact human blood group substances have both Lewis^a and Lewis^b activity, the isolated oligosaccharides are inactive as inhibitors in the Lewis system.

The study of the activity of oligosaccharides isolated by partial acid hydrolysis has afforded much information concerning the structure of antigenic determinants in blood group substances (Kabat, 1956; Morgan, 1960; Watkins, 1964). Recently the use of alkaline hydrolysis has enabled the activity of fucose-containing oligosaccharides to be studied for the first time. Schiffman *et al.* (1964a,b) isolated fractions from human A, B, and H substances and showed that some of them were many times more active as inhibitors of precipitation than the most active oligosaccharides previously obtained by partial acid hydrolysis. Morgan and his co-workers have also used alkaline hydrolysis and have isolated active trisaccharides from H (Rege *et al.*, 1964a) and Lewis^a (Le^a) substances (Rege *et al.*, 1964b) and active tetrasaccharides from human A and B substances (Painter *et al.*, 1965). Yosizawa (1961, 1962) isolated four oligosaccharides by hydrazinolysis of hog mucin blood group substances and studied their activities (Yosizawa and Miki, 1962); none of these four oligosaccharides, however, contained fucose.

The preceding papers (Lloyd *et al.*, 1966; Lloyd and Kabat, 1964) in this series described the isolation, purification, and structure of some oligosaccharides isolated from human or hog A, B, and H substances using the sodium hydroxide–sodium borohydride method of Schiffman *et al.* (1964a). The ability of these oligosaccharides to inhibit precipitation and hemagglutination has now been studied.

Materials and Methods

Inhibitors. The nonfucose-containing oligosaccharides have been described previously (Schiffman *et al.*, 1964 a,b). The fucose-containing oligosaccharides are described in the preceding paper (Lloyd *et al.*, 1966); refer to that paper for symbols used. Fraction AR_{IM5} 1.0 contains a little inert, noncarbohydrate material and possibly a small amount of another component. Fractions AR_{IM5} 2.5a and 2.5b also contain some inert material. Lacto-*N*-fucopentaose II and lacto-*N*-difucohexaose I were gifts from Professor R. Kuhn; their structures are given in Figure 1.

Antisera. Human anti-A (59–113) and anti-B (307_2) sera have been described previously (Schiffman *et al.*, 1962; Allen and Kabat, 1959). Anti-H lectin was prepared by extracting *Ulex europeus* seeds with saline

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TABLE I: Inhibition of A–anti A and B–anti B Hemagglutination by Oligosaccharides.

Substance	A Activity[a]		Substance	B Activity[a]	
	μg/ml	μmoles/ml		μg/ml	μmoles/ml
MSS AD R_{IM5} 1.0	>5,150	>5	Beach BR_{IM5} 1.2	>7,000	>7
MSS AD R_{IM5} 2.5a	2,320	2	Beach BR_L 0.44	1,300	2
MSS AD R_{IM5} 2.5b	2,360	3	α-D-Gal-(1→3)-D-Gal	>5,000	>15
MSS AR_L 0.52	1,780	2	α-L-Fuc-(1→2)-D-Galactitol	>10,800	>32
Hog AR_L 0.52	1,670	2	B substance (Beach C_6H_5OH insol)	20	...
α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-D-GNAc	9,000[b]	15			
α-L-Fuc-(1→2)-D-galactitol	>10,800	>32			
A substance (MSS 0–10% ppt)	2	...			

[a] Minimum amount of substance giving inhibition. [b] Gave partial inhibition at this concentration.

(10% solution) (Schiffman *et al.*, 1964a). Eel anti-H serum was kindly provided by Dr. G. F. Springer and we are grateful to Professor W. T. J. Morgan for supplying the rabbit and human anti-H sera (antiserum Tomlinson; *cf.* Watkins and Morgan, 1954).

Immunochemical Methods. Precipitin inhibition studies were carried out on a microscale (1–4 μg of N) as described previously (Schiffman *et al.*, 1964a). A, B, and H hemagglutination inhibition assays were carried out using a microtitrator (Cooke Engineering Co., Alexandria, Va.). Material to be tested for anti-Lewis inhibiting activity was mixed with an equal volume (5 μl) of antiserum and incubated at room temperature for 30 min. Another equal volume of freshly washed 2% saline suspension of ficin-treated (Haber and Rosenfield, 1957) red cells was then added. The presence or absence of agglutination was determined after the final mixture had incubated for 1 hr at 15°. Under these conditions and with substitution of saline for inhibitor, anti-Le^a and two examples of anti-Le^b, P72600 and 10054, had a 1:8 titer of specific agglutinins.

Experimental Section and Results

The ability of fucose-containing and nonfucose-containing oligosaccharides to inhibit the precipitation of A substance by human anti-A is shown in Figure 2. The best inhibitors are AR_L 0.52 and AR_{IM5} 2.5a and AR_{IM5} 2.5b. As demonstrated by Schiffman *et al.* (1964a) using a less pure sample of AR_L 0.52 (A_3), this fucose-containing pentasaccharide is much more active than the most active nonfucose-containing oligosaccharide previously isolated (A_5II; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962). However, the oligosaccharide AR_{IM5} 1.0 with a similar basic structure but having an additional fucose residue has considerably less A activity than AR_L 0.52 and less even than

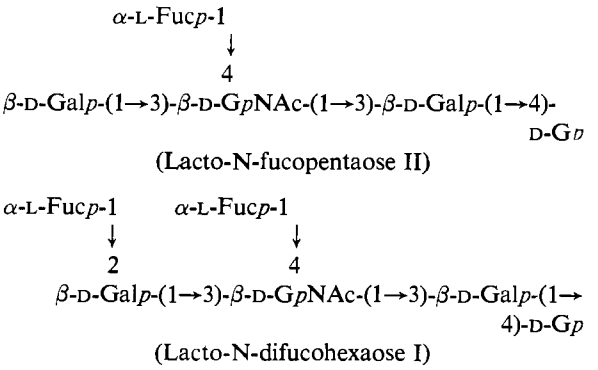


FIGURE 1: Structure of two milk oligosaccharides.

the nonfucose-containing trisaccharide, A_5II (α-D-GalNAc[1]-(1→3)-β-D-Gal-(1→3)-D-GNAc). Oligosaccharides from B (Beach BR_L 0.44) and H (Hog H R_L 0.75) have almost no inhibitory power in this system in the range studied.

The inhibition of B–anti B precipitation is shown in Figure 3. Again the reduced pentasaccharide (Beach BR_L 0.44) with a single fucose has the highest activity and is considerably more active than the B active disaccharide α-D-galactosyl-(1→3)-D-galactose. The difucose-containing hexasaccharide (Beach BR_{IM5} 1.2) is much less active while AR_L 0.52 and HR_L 0.75 are both relatively inactive. The oligosaccharides were also tested for their capacity to inhibit hemagglutination of A and B red cells. The results are given in Table I.

The ability of oligosaccharides to inhibit the hemagglutination of O cells has been studied with a variety of anti-H reagents and the results are given in Table II. The specificity of the eel anti-H serum is markedly different from the other three. In the eel system α-L-fucosyl-(1→2)-D-galactitol and L-fucose were active as inhibitors and the two tetrasaccharide samples (JS and Hog R_L 0.75) were less active. The rabbit and human anti-H sera and the *Ulex* lectin were not inhibited by the mono- and disaccharides but were

[1] Abbreviations used: GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GNAc, 2-acetamido-2-deoxy-D-glucopyranose; Gal, D-galactopyranose.

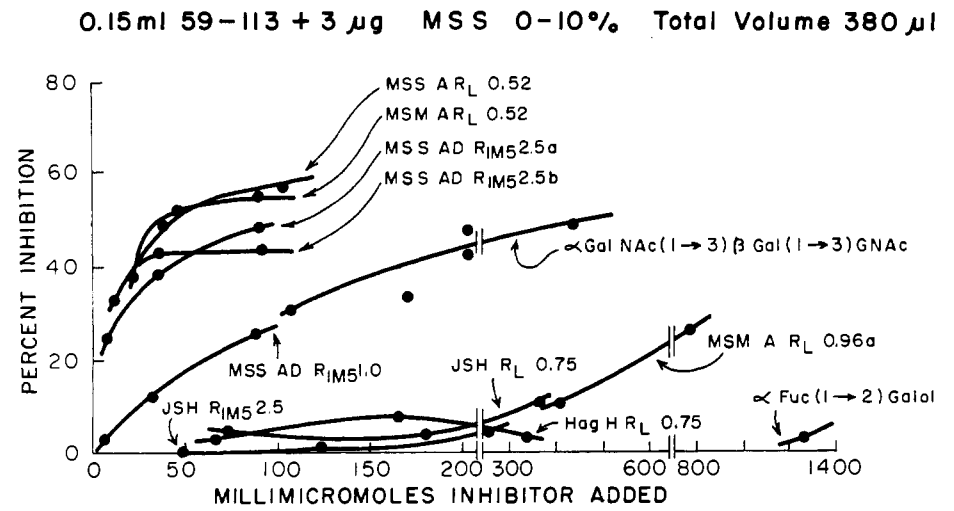


FIGURE 2: Inhibition by oligosaccharides of A–anti A precipitation.

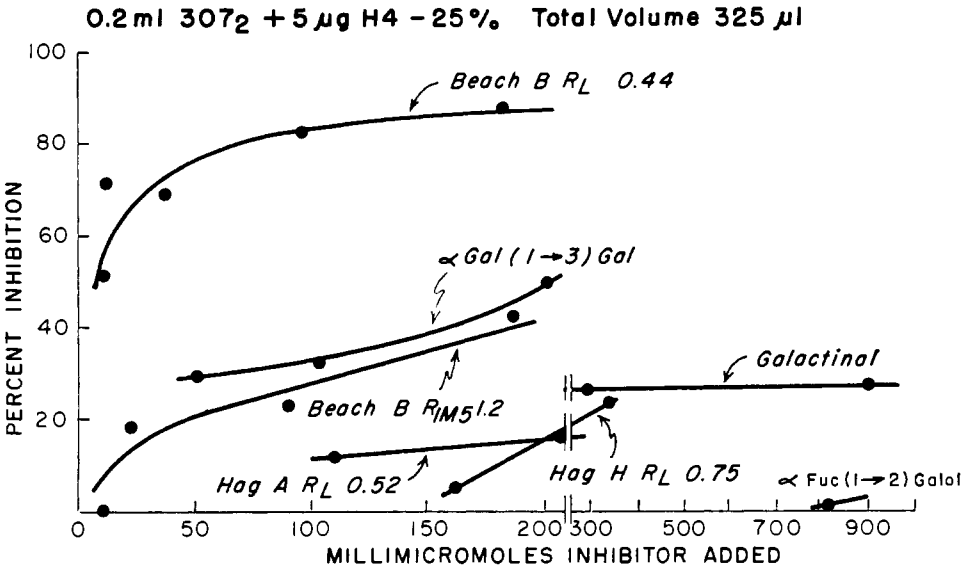


FIGURE 3: Inhibition by oligosaccharides of B–anti B precipitation.

strongly inhibited by the tetrasaccharide H R_L 0.75. In each case the difucose oligosaccharide (JS H R_{IM5} 2.5) was less active than the monofucose oligosaccharide although in the human system the difference was less marked.

The specificity of eel anti-H has recently (R. E. Rosenfield, unpublished data, 1965) been shown to differ in another respect. Type O red cells that are I-negative and i-positive (newborn or rare adults) are not agglutinated by eel serum but are by the other sources of anti-H. *Ulex* anti-H showed the least ability to distinguish between type O cells that varied in their I–i status.

The power of the oligosaccharides to inhibit hemagglutination by Le^a and Le^b antisera is shown in Table III. None of the oligosaccharides isolated inhibited although the original human A, B, and H substances themselves had quite high activity. The two milk oligosaccharides, lacto-*N*-fucopentaose II and lacto-*N*-difucohexaose I, which are inhibitors of Le^a and Le^b, respectively, were included as reference compounds.

Discussion

By partial acid hydrolysis two pairs of active nonfucose-containing trisaccharides have been isolated from A and B substances (Cheese and Morgan, 1961; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962; Painter *et al.*, 1963).

TABLE II: Blood Group H Activity of Oligosaccharides Determined with Various Anti-H Reagents.

Substance	Min Amount of Substance Giving Inhib							
	Ulex Anti-H		Eel Anti-H		Human Anti-H		Rabbit Anti-H	
	μg/ml	μmoles/ml	μg/ml	μmoles/ml	μg/ml	μmoles/ml	μg/ml	μmole/ml
L-Fucose	>7,500	...	560	3.4	>7,500	...	>7,500	...
α-L-Fuc-(1→2)-D-galactitol (MSM)	>10,000	...	190	0.6	>10,000	...	>10,000	...
α-L-Fuc-(1→2)-D-galactitol (Hog)	>10,000	...	200	0.6	>10,000	...	>10,000	...
JS H R_L 0.75	220	0.3	1,560	2.4	370	0.6	580	0.8
Hog H R_L 0.75	280	0.4	2,120	3.2	350	0.5	510	0.8
JS H R_{IM5} 2.5	10,000	12	>8,500	...	1,280	1.6	>8,500	...
JS H substance	13	...	60	...	60	...	8	...
Hog H substance	3	...	7	...	30	...	2	...
Hog A R_L 0.52	>8,000	...	>8,000	...	>8,000	...	>8,000	...
Beach B R_L 0.44	>7,400	...	>7,400	...	>7,400	...	>7,400	...

Type 1 A
α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-D-GNAc

Type 2
α-D-GalNAc-(1→3)-β-D-Gal-(1→4)-D-GNAc

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

Type 2
α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-GNAc

These trisaccharides are considered to be partial structures for two types of A and B antigenic determinants. From A, B, H, and Le^a substances both β-D-Gal-(1→3)-D-GNAc and β-D-Gal-(1→4)-D-GNAc typical of types 1 and 2 structures have also been isolated (Painter *et al.*, 1963; Schiffman *et al.*, 1962).

Of the oligosaccharides isolated by alkaline hydrolysis of A substance, AR_L 0.52 (Figure 1) is the most active and is at least 10 times more active as an inhibitor of A–anti A precipitation than the nonfucose-containing trisaccharide: α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-D-GNAc. The terminal nonreducing portion of this oligosaccharide has the same structure as the type 2 A-active trisaccharide but with a fucose residue as a side chain on the C-2 position of the galactose. Except for the presence of an additional residue (R) at the reduced end, this oligosaccharide is identical with the A-active tetrasaccharide recently isolated by Painter *et al.* (1965). Two other oligosaccharides, AR_{IM5} 2.5a and AR_{IM5} 2.5b, have comparable activities. They have the same basic structure except that they have galactitol at the reduced end, and AR_{IM5} 2.5a has the type 1,β-D-Gal-(1→3)-D-GNAc, linkage in its chain. The structure of hexasaccharide AR_{IM5} 1.0 resembles AR_L 0.52 but it has a second fucose side chain substituted on the *N*-acetylglucosamine. It is considerably poorer than AR_L 0.52 as an inhibitor of both hemagglutination and precipitation in the A–anti A system.

Two similar oligosaccharides have also been isolated from B substance although of course these are terminated at the nonreducing end by a D-galactose residue rather than an *N*-acetyl-D-galactosamine. The same pattern of activity was observed in that the monofucooligosaccharide (BR_L 0.44) is considerably more active than the fucose-free α-D-galactosyl-(1→3)-D-galactose and the difucooligosaccharide (BR_{IM5} 1.2) is less active as an inhibitor of B–anti B precipitation and hemagglutination than both these compounds (Figure 3 and Table I).

From H substance two corresponding oligosaccharides have been isolated which are similar in structure to the two pairs of A and B oligosaccharides except that they lack the terminal *N*-acetylgalactosamine or galactose residue and, therefore, have only L-fucose in a terminal nonreducing position. As is shown in Table II HR_L 0.75 is considerably more active than HR_{IM5} 2.5 in all anti-H systems studied.

Thus with three blood group substances the substitution of the oligosaccharide chains by a single fucosyl residue (onto a galactose) is responsible for a large increase in their activities as inhibitors of precipitation or hemagglutination.[2] This is in contrast to the findings of Painter *et al.* (1965) that the A and B tetrasaccharides described by them are only two to four times more active as inhibitors of hemagglutination than the oligosaccharides having no fucose. With the substitution of a second fucose, on the adjacent *N*-acetylglucosamine, this effect is lost and the oligosaccharides are less active, although it could be imagined that such a structure might represent a more complete determinant. It is curious also that although the presence

[2] Springer *et al.* (1964b) also found that a monofucose-containing penta- or hexasaccharide from *Escherichia coli* was 20 times as active in the inhibition of B–anti B as α-D-galactosyl-(1→3)-D-galactose and a heptasaccharide (also containing one fucose) was four times as active as this disaccharide.

TABLE III: Blood Group H and Lewis Activities of Crude and Purified Oligosaccharides.

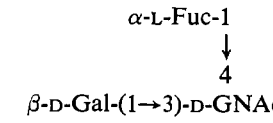
Substance	Min Amount of Substance Giving Inhib (μg/ml)			
	H (*Ulex*)	Le^a	Le^b (Antiserum 1)	Le^b (Antiserum 2)
MSS 0–10% ppt (A substance)	1,000	13	<100, 1	1
MSM (A substance)	990	22	<99, 16	2
MSM Dial I	>5,000	>5,000	>5,000	...
MSM Dial II	5,000	>5,000	5,000	...
MSM Dial III	5,000	>5,000	1,250	...
MSM Nondial	5,000	350	210	...
MSS A R_L 0.52 + MSM A R_L 0.52	>10,000	>10,000	5,000	...
MSS ADR_{IM5} 1.0	>19,700	9,800	4,900	>1,970
Beach C_6H_5OH insol (B substance)	190	6–25	2–4	4–8
Beach B R_{IM5} 1.2	>17,600	8,800	17,600	1,760–17,600
Beach B R_L 0.44	>20,000	5,000	20,000	2,500
JS C_6H_5OH insol (H substance)	7	6	0.1–0.2	0.2
JS H R_{IM5} 2.5	>20,000	>20,000	5,000	5,000
JS H R_L 0.75	260	>21,000	>21,000	>21,000
Lacto-*N*-fucopentaose II	>1,780	44	>1,780	900
Lacto-*N*-difucohexaose I	>1,860	190	45	45

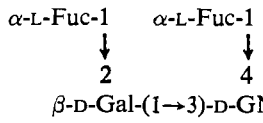
of a fucose on the galactose of the A and B oligosaccharides is responsible for a large increase in their inhibiting activity, fucose oligosaccharides lacking terminal *N*-acetylgalactosamine and D-galactose have no activity in the A and B systems over the range studied. It is possible that the antibody combining site does not include the fucose residue and that the function of this sugar on the galactose is rather to hold the remainder of the molecule in a preferred orientation. In H substance L-fucose assumes a dominant role in specificity but this is only to be expected since it is the terminal nonreducing end. The recent findings (Beychok and Kabat, 1965) by optical rotatory dispersion that oligosaccharides containing *N*-acetylhexosamines exhibit a preferred conformation in solution and that substitution on various carbons alters the conformational possibilities makes this a very attractive hypothesis for explaining differences in antigenic specificity. In the case of blood group oligosaccharides a preferred conformational state determined by substitution of fucose on the oligosaccharide chains would at once account for the increased activity of the di- and trisaccharides over the monosaccharides, shown for the fucose-free oligosaccharides, as well as for the increased activity caused by substitution of fucose on the galactose residues. It would also account for the decreased activity caused by the presence of a second fucose, substituted onto the *N*-acetylglucosamine. Although preferred conformations in solution have been shown thus far only for hexosamine-containing oligosaccharides because of the acetamido Cotton effect they may equally well exist for all oligosaccharides.

The H activity of the oligosaccharides has been studied with a number of anti-H reagents. Three of these, human anti-H, rabbit anti-H, and *Ulex* lectin have rather similar specificities but the eel anti-H is different. Springer *et al.* (1964a) have shown that the antibody site in eel anti-H is small and directed toward the L-fucose residue, probably even toward only a portion of this sugar. In agreement with this L-fucose itself is more active than the tetrasaccharide (H R_L 0.75) from human and hog substances. α-L-Fucosyl-(1→2)-D-galactitol is slightly more inhibitory than L-fucose, suggesting that the antibody combining site extends at least as far as the glycosidic carbon of the fucose residue (*cf.* Rege *et al.*, 1964a). Both L-fucose and α-L-fucosyl-(1→2)-D-galactitol are inactive as inhibitors in the human and rabbit and *Ulex* anti-H systems. In these systems the reduced tetrasaccharide H R_L 0.75 is the most active inhibitor and the difucosyloligosaccharide (H R_{IM5} 2.5) is less active, particularly against *Ulex* and rabbit anti-H. With *Ulex*, rabbit, and eel anti-H, H R_L 0.75 is about 40 times as active as H R_{IM5} 2.5 but only four times as active using human anti-H serum.

Although both the AR_L 0.52 and BR_L 0.44 pentasaccharides have a fucose linked α-(1→2) to galactose as in the H active HR_L 0.75, the presence of the terminal *N*-acetyl-D-galactosamine in the A and D-galactose in the B oligosaccharides must block the access of the anti-H antibody site to the fucosylgalactose portion of the determinant thus masking the H specificity. Both A and B determinants have an underlying H structure in agreement with the finding that H activity is produced when *N*-acetylgalactosamine is removed enzymatically from A substance (Iseki and Masaki, 1953; Watkins, 1962; Marcus *et al.*, 1964) and when D-galactose is removed from B substance (Watkins and Morgan, 1956; Iseki *et al.*, 1959; Zarnitz and Kabat, 1960; Watkins *et al.*, 1962).

Although the three human blood group substances used in this study have both Le^a and Le^b activity, none of the oligosaccharides so far isolated have Lewis activity (Table III). Inhibition studies (Watkins and Morgan, 1957, 1962) using the milk oligosaccharides isolated by Kuhn and co-workers indicated that Le^a activity resides in the first structure while the second





structure is responsible for Le^b activity. The importance of the branched trisaccharide in Le^a activity has recently been confirmed by the isolation of this oligosaccharide by alkaline hydrolysis of Le^a substance (Rege *et al.*, 1964b). Oligosaccharide H R_{IM5} 2.5 resembles the Le^b active structure except that the linkage is β-D-Gal-(1→4)-D-GNAc and the linkage of the fucose to the *N*-acetylglucosamine is not known. This oligosaccharide is inactive as an Le^a and Le^b inhibitor, indicating that the positions of substituents on the *N*-acetylglucosamine residue is important. The A and B difucosyloligosaccharides, which are also inactive in these systems, have the same structure as H R_{IM5} 2.5 but are additionally substituted with *N*-acetyl-D-galactosamine and D-galactose, respectively, at the nonreducing end.

So far no oligosaccharides have been isolated which have fucose substituted on the *N*-acetylglucosamine but not on the galactose residues as would be expected of an Le^a active oligosaccharide. It is probable that the Le^a and Le^b activity of the original A, B, and H substances is due to the presence of biosynthetically uncompleted chains (Watkins and Morgan, 1959). Since none of the oligosaccharides produced by the action of alkaline borohydride have Le^a or Le^b activity, these chains may be particularly susceptible to alkali and would, therefore, be destroyed.

Whether the difucose-containing oligosaccharides from A, B and H represent additional A, B, and H determinants of specificity somewhat different than those of the respective monofucose-containing oligosaccharides remains to be determined. The latter do not give 100% inhibition with many anti-A and anti-B sera (*cf.* Figure 2, and Schiffman *et al.*, 1964a).

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