[CONTRIBUTION FROM THE DEPARTMENTS OF NEUROLOGY AND MICROBIOLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL]

Immunochemical Studies on Blood Groups. XVII. Structural Units Involved in Blood Group A and B Specificity[1]

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An oligosaccharide fraction obtained by paper chromatography of the dialyzable material released by mild acid hydrolysis of hog blood group A substance shows the ability specifically to inhibit the precipitation of anti-A by A substance. No inhibition was obtained with a similar material from hog O (H) substance. Of the monosaccharide constituents of the blood group substances only N-acetylgalactosamine showed slight inhibition. The disaccharide galactosido-1→4-β-N-acetylglucosamine also inhibited weakly. Inhibition of precipitation of anti-B by human saliva or horse stomach B substances was produced in order of decreasing effectiveness on a molar basis by (1) raffinose, melibiose and stachyose; (2) α-methylgalactoside; (3) galactose; (4) β-methylgalactoside. A variety of other sugars did not inhibit. The inhibition data indicate that B specificity is determined by an oligosaccharide with a terminal non-reducing galactose linked to the next sugar in 1→6-α-galactosidic linkage; this second sugar is probably N-acetylglucosamine. Comparison of the molar concentrations required for inhibition in the blood group systems as compared with those for dextran–anti-dextran suggests that the oligosaccharide side chains determining blood group specificity are probably no larger than tri- to penta- or hexasaccharides.

Recent studies from this Laboratory[2] have shown that the dialyzable materials liberated by mild acid hydrolysis (*p*H 1.5–2.0 at 100° for 2 hours) of the blood group A, O and B substances from hog and human sources have glucosamine–galactosamine ratios which correlate with their blood group specificity. In the present communication it is shown that the oligosaccharide fractions of such dialysates possess groupings which determine blood group specificity in that the oligosaccharide fraction from hog A substance inhibits the precipitation of anti-A by hog or human A substance, while a similar material from hog O substance shows no such effect on anti-A. Comparisons are made of the relative potency of various monosaccharides and oligosaccharides of known structure which inhibit the A–anti-A and the B–anti-B precipitin reactions. Inferences are then drawn as to the structure of a portion of the oligosaccharide units which determine A and B specificity.

Experimental

Dialyzable Oligosaccharides from Mild Acid Hydrolysates of Blood Group Substances.—A sample of hog mucin substance (Fr2) containing a mixture of hog A and O (H) substances as well as an A (Hog 39) and an O (H) (Hog 33) sample each from individual hog stomach linings were studied. A weighed sample of the substance was dissolved in a measured volume of dilute HCl (*p*H 1.6) to give a solution containing 10 or 15 mg. of substance per ml. and heated for 2 hours in a boiling water-bath in a tube sealed with a self sealing rubber cap (the pressure was released by puncturing the cap with a hypodermic needle). The solution was then transferred to a cellophane sausage casing and dialyzed against at least 5 changes of distilled water for 5 days. The combined dialysates were brought to *p*H 6 with dilute NaOH in the case of the hog mucin substance (Fr2) and then concentrated under nitrogen and reduced pressure at 40° and lyophilized. Since this procedure gave considerable quantities of salt, the dialysates of the other two substances were lyophilized without neutralization. The non-dialysable residues (P1) were also recovered by lyophilization.

Measured amounts of dialysate were spotted on sheets of Whatman #1 filter paper which were developed in a descending chromatogram with ethyl acetate–propanol–water (1:7:2)[3] as the solvent. After about 18 hours, the papers were removed, dried and redeveloped[4] with the same solvent mixture. After the second drying guide strips were cut off, bathed in a 2% hexane solution of aniline, dried, then bathed in a 2% solution of trichloroacetic acid in hexane, dried, and heated in an oven at 100° for 10 minutes to locate the reducing sugars as brown spots.

By reference to the guide spots the main papers were cut to obtain four bands in the case of hog mucin substance (Fr2) and three bands for hogs 39 and 33. These bands corresponded to materials of R_F 0.64, 0.32, 0.23, and a streak to the origin for Fr2. For Hog 33 and 39 the bands had R_F 0.55, 0.3, and a streak to the origin. These represented fucose and N-acetylhexosamine, galactose and glucosamine, and oligosaccharides, respectively.[5] The papers corresponding to each band were extracted with 20 ml. of water. Aliquot portions were examined directly and after hydrolysis with 2 *N* HCl for 2 hours at 100°.

Analytical Methods.—Reducing sugar was determined by the Hagedorn–Jensen method,[6a] hexosamine by the Elson–Morgan method[6b]) after evaporating samples hydrolyzed with HCl to dryness in a vacuum desiccator,[7] methylpentose by the Dische and Shettles method[8] and N-acetylhexosamine as recently described by Aminoff, Morgan and Watkins.[9] Glucosamine–galactosamine ratios were measured by the method recently described from this Laboratory.[10]

Immunochemical Studies.—The micro modification of the quantitative precipitin method of Heidelberger and MacPherson[11] was used. The capacity of various fractions obtained from the dialyzates of the blood group hydrolysates and of various monosaccharides and oligosaccharides of known structure to inhibit precipitation was measured by adding known quantities of the substance to be tested, or of saline, to a measured volume of antiserum, incubating at 37° for 30 minutes, and then adding an appropriate amount of blood group substance. After mixing, the tubes were again incubated at 37° for one hour and placed in the refrigerator for one week with mixing twice daily. The tubes were centrifuged, the precipitates washed twice in the cold with saline and analyzed for N with the Folin–Ciocalteu tyrosine reagent.[11,12] The percentage inhibition by any

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TABLE I

PROPERTIES OF DIALYSATE FRACTIONS OBTAINED ON MILD ACID HYDROLYSIS OF HOG BLOOD GROUP SUBSTANCES

	Hog mucin Fr2 A + O (H)	Hog 33 B2 O (H)	Hog 39 B2 A
Amount substance used, g.	1.08	0.385	0.392
Conditions of hydrolysis	*p*H 1.6, 100°, 2 hr.	*p*H 1.6, 100°, 2 hr.	*p*H 1.6, 100°, 2 hr.
Concn. of soln. hydrolyzed, mg./ml.	10.8	15.4	15.7
Non-dialyzable P1 fraction	0.819	0.162	0.172
Dialyzable material,[a] g.	0.26	0.22	0.22

Composition of bands obtained on paper chromatography of dialysate

Band designation	Ethyl acetate–propanol–water (1:7:2) A	B	C	D	A	B	C	A	B	C
R_F	0.64	0.32	0.23	0.0[b]	0.55	0.30	0.0[b]	0.55	0.3	0[b]
Methylpentose, mg.	55	0.4	0.6	2.1	16.2	2.1	5.8	16.4	3.1	5.9
Reducing sugar as glucose: Unhydrolyzed, mg.	53	9.0	12.9	18.3	32	24	28	33	30	34
Reducing sugar as glucose: Hydrolyzed,[c] mg.	48	8.9	22	42	36	24	50	35	31	55
Hexosamine hydrolyzed, mg.	15.5	1.6	11.2	19	22	10.6	21	22	18	26
Glucosamine/galactosamine	3.6	3.2	3.9	3.1	5.5	10.4	8.4	3.1	2.0	2.5
Equivalent N-acetylhexosamine,[d] mg.					21[e]	2.5[e]	8.2	18[e]	4.4[e]	9.6
Inhibition studies	1.5 ml. KN_1; 50μ g. Hog 30(A)—Total vol. 3.0 ml.									
Sample added, mg. reducing sugar on hydrolysis	2.2	0.4	0.8	1.7	1.8	1.2	2.5	1.7	1.5	2.7
% inhibition	0	0	17	59	2	−1	−2	−2	−2	23

[a] By difference. [b] A streak trailing from the origin toward the next faster moving band was taken. [c] After hydrolysis at 100° for 2 hours in 2 *N* HCl. [d] Maximum values of equivalent N-acetylhexosamine were obtained after 4 minutes' heating with Na_2CO_3.[9] [e] Analyses by Mr. Joel Goodman.

concentration of inhibitor was computed from the difference in the amount of specific precipitate formed in the presence of the inhibitor and that formed in saline, a quantitative adaptation[13,14] of the qualitative hapten inhibition procedure developed by Landsteiner[15,16] to the quantitative measurement of the degree of inhibition.

Antisera to hog A substance and to human A saliva were used. The antisera to hog A substance, KN_1 and KN_2[17] were prepared by injection of individuals of group B with hog A substance.[18] The antiserum to human saliva A substance, PM_4, was prepared by injection of purified human saliva A substance into an individual of blood group B.[19] The antiserum to B substance, Als., described in reference 20, was from a woman of group O who had a marked post-partum rise in anti-B following delivery of a B infant with erythroblastosis and kernicterus; it was kindly provided by Drs. R. E. Rosenfield and A. B. Gutman of Mount Sinai Hospital. For the oligosaccharide inhibition studies, 1.5 ml. of KN_1 (30 μ g. AbN), 1.0 ml. of KN_2 (23 μ g. AbN) and PM_4 (22 μ g. AbN) and 0.5 ml. of a 2 → 3 dilution of Als. (30 μ g. AbN) were used; the total volumes were 3.0 ml. for KN_1, 2.5 ml. for KN_2 and PM_4 and 2.0 ml. for Als.

In addition to the fractions obtained by mild acid hydrolysis, dialysis and paper chromatographic separation of the hog A and O substances, various mono- and oligosaccharides were used in the inhibition studies. Among these were α-([α]^{20}D +179°) and β-([α]^{20}D −0.5°) methylgalactopyranosides, melibiose ([α]^{20}D +142.3° (final)),[21] and neolactose ([α]^{20}D +35.5°)[22] kindly supplied by Dr. Nelson K. Richtmyer, National Institutes of Health, stachyose by Dr. Dexter French,[22a] Iowa State College, galactosido-1→4-β-N-acetylglucosaine[23] by Dr. R. M. Tomarelli, Wyeth Laboratories, and galactosamine by Dr. Karl Meyer. Samples of melibiose [α]D +143°, raffinose [α]^{26}D +125°, lactose [α]^{26}D +55.5°, and galactose [α]^{26}D +80° (Eastman Kodak Company), N-acetylglucosamine [α]^{22}D +39.5° 16 hours, fucose [α]D −77° (Pfanstiehl Company) and ordinary commercial sucrose were used. N-Acetylgalactosamine was prepared as described by Roseman.[24]

Because of the limited amounts of serum and oligosaccharide available, determinations in any individual experiment could not be carried out in duplicate except for the serum samples to which only antigen and no inhibitor was added. However, with the A–anti-A and B-anti-B systems each substance was tested as an inhibitor in several independent experiments so that from 4 to 7 assays for inhibiting potency on each substance were carried out. An indication of the experimental error may be obtained by comparing the quantity of antibody precipitable by the same amount of antigen from a given antiserum in four separate experiments in duplicate in which values were 26.7, 27.7, 29.8 and 30.1 μ g. N. While, in general, the results in single experiments give closer agreement, if one admits the occasional possibility that a variation of ±1 to 2 μ g. of antibody N could occur in the analysis without inhibitor and a variation of the same amount in the presence of the inhibitor differences of up to ±10% in the value per cent. inhibition could result, leading occasionally to slight negative values for per cent. inhibition. Thus in testing the inhibiting power of lactose in the B–anti-B system values of 22.9 μ g. N without lactose and 24.5 μ g. N with lactose were obtained. This calculates to −6% inhibition; on repetition in a completely independent experiment with the same amount of lactose a value of 3% inhibition resulted; both of these are within experimental error.

Results

The data in Table I confirm and extend previous findings[2] on the effects of mild acid hydrolysis on hog A and O substances. The fast moving spot (band A) can now be seen to contain N-acetylhexosamine in addition to the fucose previously identified. This may also be seen retrospectively from the data in reference 2 in which the fucose

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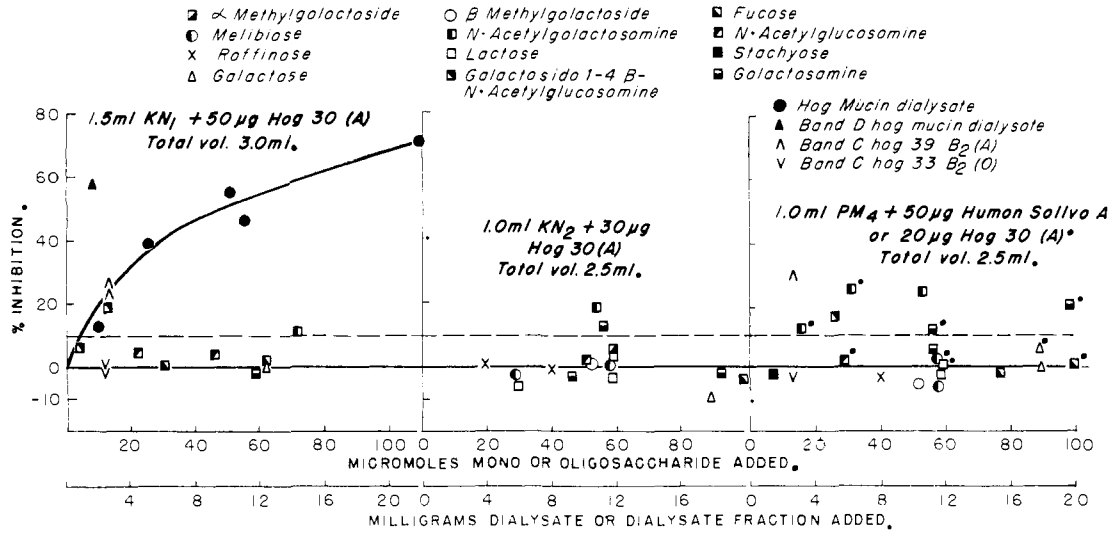

Fig. 1.—Inhibition of A–anti-A precipitation by various sugars and by dialysates and dialysate fractions from mild acid hydrolysis of hog blood group A and O substances. Data on the sugars are plotted in micromoles while those of the dialysate and dialysate fractions are plotted in terms of mg. of substance based on hydrolyzed reducing sugar values.

spot showed increased hexosamine[6] after hydrolysis. The second band (B) represents galactose and hexosamine while the slower moving and immobile materials (band C and D for hog mucin Fr2 and band C for the individual hog substances) consist of polymerized materials. Paper chromatographic study of hydrolysates of Fr2 band D and band C of hog 33 B2 and 39 B2 gave spots corresponding in R_F to galactose, glucosamine and fucose, the latter in small amount. The oligosaccharide-containing bands probably represent a mixture of materials of differing composition and degrees of polymerization.

The most striking finding, however, is that the oligosaccharide-containing fraction from blood group A substance specifically inhibits precipitation of anti-A by A substance, while comparable amounts of the same fraction from a hog O substance do not inhibit nor do bands A and B from both the hog A and O preparations (Table I and Fig. 1). This inhibition occurred both with human antiserum to hog A substance (KN_1) and with human antiserum to human saliva A substance (PM_4). The much greater inhibiting power per unit weight of the total dialysate and of band D from hog mucin, a mixture of A and O substances, as compared with the individual hog A substance, together with the finding that the hydrolysis had been much more extensive in the latter instance suggests that active material in the hog A dialysate was diluted with a much higher proportion of oligosaccharides derived from the main polysaccharide chain rather than from the specific side chains determining blood group specificity.

Inhibition studies were carried out to determine whether any known monosaccharides or oligosaccharides were capable of inhibiting A–anti-A precipitation. These are also given in Fig. 1. Of the various sugars used only N-acetylgalactosamine and the galactosido 1→4-β-N-acetylglucosamine showed some inhibition. Galactosamine showed weak inhibition with anti-human A (PM_4) but not with anti-hog A, while all the other sugars tested did not inhibit within experimental error. The extent of inhibition of the sugars which did inhibit was much less than that of the hog mucin dialysate or its oligosaccharide fraction. With serum PM_4 the oligosaccharide fraction from the A hog was a much better inhibitor than these two sugars; larger amounts of the individual hog A dialysate were not available for testing. In Fig. 1 the inhibiting capacities of the known sugars are plotted as micromoles used in the test, while the dialysate fractions are plotted in milligrams. The scales, however, are such that they correspond roughly to micromoles of a monosaccharide. The dotted line indicates the outside limit of experimental error (*cf.* 14). Since the analytical data in Table I indicate that the oligosaccharide-containing band has an average degree of polymerization of 2 or 3 sugar units, the data in Fig. 1, if plotted on a molar basis, would indicate that even smaller molar concentrations were needed to produce inhibition of the A–anti-A reaction. It is especially significant that, as previously reported,[2] N-acetylgalactosamine occurs in appreciable amounts in the dialyzable fractions derived by mild acid hydrolysis of the hog A substance and that this substance should itself be an inhibitor.

Determinations of equivalent N-acetylhexosamine in fraction C of hog 39 indicate the presence of 9.6 mg. of equivalent N-acetylhexosamine expressed as N-acetylglucosamine. From the total hexosamine and the glucosamine–galactosamine ratio, there would be about 7.4 mg. of galactosamine or 9.1 mg. of N-acetylgalactosamine in this fraction. Aminoff, Morgan and Watkins[9] report that N-acetylgalactosamine gives only about 23% of the color of an equal amount of N-acetylglucosamine so that the 9.1 mg. of N-acetylgalactosamine were it present, as a reducing end group, would give only 2.1 mg. of equivalent N-acetylhexosamine. These findings show that most of

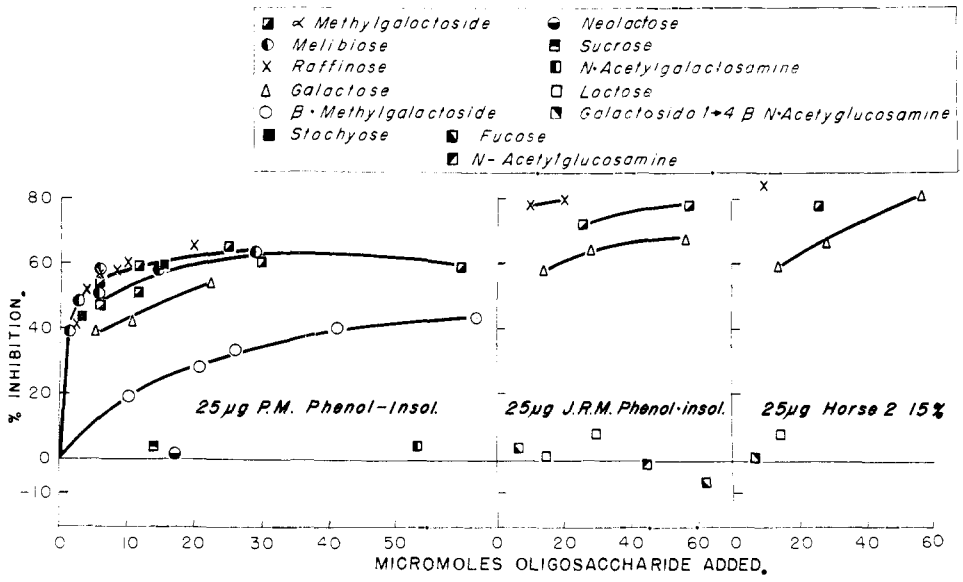

Fig. 2.—Inhibition of B-anti-B precipitation by various oligosaccharides.

the N-acetylhexosamine color is due to reducing groups of N-acetylglucosamine. Since free N-acetylgalactosamine inhibits A–anti-A precipitation while free N-acetylglucosamine does not, it may well be that a terminal non-reducing N-acetylgalactosamine is important in A specificity. The dialysate of the hog O substance appears to contain comparable amounts of terminal reducing N-acetylhexosamine although only very small amounts of N-acetylgalactosamine are present.

In the case of the blood group B substance, even more insight into the groupings determining its specificity was obtained by inhibition studies although the products obtained by mild acid hydrolysis have not as yet been tested. Figure 2 shows that raffinose, melibiose, stachyose, α-methylgalactoside, galactose and β-methylgalactoside are excellent inhibitors of B–anti-B precipitation, while other sugars present in the blood group substances, fucose, N-acetylglucosamine, N-acetylgalactosamine as well as lactose, neolactose, galactosido 1 → 4-β-N-acetylglucosamine, sucrose, etc., do not inhibit. The extent of inhibition of those substances which inhibit reaches as high as 80% for the reaction of anti-B with either human saliva B or horse stomach B substances in contrast to the A–anti-A precipitation in which the maximum inhibition achieved with known sugars was only about 20%.

Among the sugars which inhibit B–anti-B precipitation, raffinose, melibiose and stachyose were the best inhibitors and were equally effective on a molar basis. The next most effective inhibitor was α-methylgalactoside, galactose was less potent and β-methylgalactoside least. It is evident that the three best inhibitors contain a terminal non-reducing 1→6-α-galactopyranoside linkage attached to a second sugar ring. Replacement of this second ring by hydrogen as in α-methylgalactoside reduces the inhibiting power slightly; elimination of the galactosidic linkage causes further loss of activity and a substantial additional reduction in inhibiting power is imposed by the substitution of a β-galactosidic linkage as in β-methylgalactopyranoside. It thus appears that a terminal non-reducing 1→6-α-galactopyranosidic linkage is an important determinant of blood group B specificity.

In two of the three best inhibitors, melibiose and raffinose, the sugar to which the terminal 1→6-α-galactosidic unit is attached, namely, glucose, is not a constituent of the blood group substance. In stachyose, however, the second sugar is also a galactose. The use of stachyose as an oligosaccharide with two galactosidic units in 1→6-α-linkage was suggested by Mr. Peter Allen. It would be anticipated that if the terminal non-reducing galactosidic group in the blood group B substance were attached to a second galactose, a substantial increase in inhibiting power per mole over raffinose or melibiose would be expected.[14] Since this did not occur, it is probable that a galactose unit can be excluded as the second unit. The finding[2] that glucosamine is the only hexosamine split off by mild acid hydrolysis of B substances together with this latter observation on stachyose would suggest that the sugar to which the terminal non-reducing galactose residue is attached in 1→6-α-linkage is N-acetylglucosamine. Galactosido 1→6-α-N-acetylglucosamine is not yet available for study.

The relation of the specificity of the B substance to a 1→6-α-galactopyranoside linkage could also be demonstrated by the classical hemagglutination inhibition test with B erythrocytes and anti-B. Table II shows the results of adding 2 mg. of various oligosaccharides to 0.1 ml. of serial dilutions of the anti-B serum, incubating at 37° for ½ hr. and adding 0.1 ml. of 4% washed B erythrocytes. Raffinose, melibiose and galactose produce some inhibition of hemagglutination while gentiobiose, sucrose, fucose, N-acetylglucosamine and lactose show no inhibition within experimental error. The precision of this technique, however, would not permit conclusions as to the relative potency of the various inhibiting substances so that it has much

TABLE II

EFFECT OF VARIOUS SUGARS ON HEMAGGLUTINATION OF B ERYTHROCYTES BY ANTI-B

Procedure: 2 mg. of sugar in saline added in 0.2 ml. to 0.1 ml. of antiserum, incubate 30 min. at 37°; add 0.1 ml. of 4% washed human B erythrocytes, incubate 1 hr. at 37°, centrifuge lightly and read. Degrees of hemagglutination are graded from − to ++++.

Sugar added, 2 mg. each	0.1 ml. of antiserum Als. diluted 1 to 20	40	80	160	320	640	1280
None	++++	++++	++++	+++	++	+±	−
Raffinose	++	+±	+±	+	−	−	−
Melibiose	++	+	+	±	−	−	−
Galactose	++	++	+	+	−	−	−
Gentiobiose	++++	++++	++++	+++	++±	+±	−
Lactose	++++	++++	+++±	++±	+	±	−
Fucose	+++±	++++	++++	+++	++±	+±	−
N-acetylglucosamine	++++	++++	++++	+++±	++	±	−
Sucrose	++++	++++	++++	+++±	++±	+	−

more limited significance than the quantitative precipitin procedure in elucidating structural relationships. Hemagglutination inhibition tests have also been used by Morgan and Watkins[25] in determining structural units involved in the hemagglutination of O erythrocytes by anti-O (H) sera and of A or B erythrocytes by the plant seed agglutinins.

Discussion

The data presented provide the first definitive evidence that the specific immunological ability of the blood group A and B substances to react with homologous isoantibody is associated with the carbohydrate rather than with the amino acid portion of these materials. This is especially evident from the findings that oligosaccharides containing a terminal non-reducing 1→6-α-galactopyranoside linkage give as much as 80% inhibition of B–anti-B precipitation (Fig. 2) and also inhibit B–anti-B hemagglutination (Table II). In the A–anti-A system it is indicated by the finding that N-acetylgalactosamine and galactosido 1→4-β-N-acetylglucosamine[23] also inhibit, but the extent of the inhibition is much less marked.

That the dialyzable oligosaccharide fraction from mild acid hydrolysis of hog A substance specifically inhibits A–anti-A precipitation while a similar fraction from hog O substance fails to inhibit, provides strong support for the recent hypothesis[2] put forward from data on the glucosamine–galactosamine ratios of such dialysates that blood group A, B and O specificity was determined by oligosaccharide side chains and that these side chains were split off by mild acid hydrolysis. Since N-acetylgalactosamine is the only monosaccharide which inhibits A–anti-A precipitation and inasmuch as only the dialyzable and inhibitory oligosaccharide fraction C of the hog A substance (hog 39 B_2) contains appreciable N-acetylgalactosamine (Table I), it may be inferred that this sugar may be the non-reducing terminal group of the blood group A specific oligosaccharide. Consistent with this is the finding that much of the N-acetylhexosamine found in fraction C of both hog A and O substances is terminal reducing N-acetylglucosamine. It is also in agreement with the data of Morgan and Watkins[25] that N-acetylgalactosamine inhibited the hemagglutination of A cells by A-specific extracts of plant seeds such as Lima beans and *Vicia cracca* and indicates that at least part of the same groupings on the blood group A substance may be responsible both for reaction with anti-A and with the plant seed agglutinins with A specificity.

The finding that galactosido 1→4-β-N-acetylglucosamine also shows some inhibition of A–anti-A precipitation is difficult to interpret. Since the degree of inhibition is relatively slight and no greater than that of N-acetylgalactosamine, it probably does not constitute a unit present *per se* in the specific A oligosaccharide side chain. Perhaps the slight inhibiting power is related to the 1→4-β-linkage attached to a reducing N-acetylglucosamine since lactose showed no inhibition.

In the case of the B–anti-B reaction the structure of a larger portion of the specific oligosaccharide side chain has been elucidated and it has been suggested that the galactosido 1→6-α-N-acetylglucosamine probably makes up the non-reducing terminal two units of the B specific oligosaccharide.

Morgan and Watkins[25] reported weak inhibition by melibiose and raffinose of hemagglutination of B erythrocytes by *Sophora japonica* extracts (which agglutinate both A and B cells), but these oligosaccharides were less effective than N-acetylgalactosamine and lactose, so that this system would not appear to shed any light on the B specific groupings but probably involves other structures common to the A and B substances.

There is abundant immunochemical evidence to support the inference that the N-acetylgalactosamine and the galactosido 1→6-α linkage exert their substantial inhibiting effects largely because they occur at the ends of the A and B oligosaccharide chains. Studies of Landsteiner and van der Scheer[26] on the specificity of azo proteins in which small polypeptide chains had been introduced onto the protein molecule showed that the terminal amino acid played the dominant role in specificity, with the other amino acids of the chain playing a lesser role. Goebel, Avery and Babers[27] coupled disaccharides such as cellobiose, gentiobiose, lactose and maltose to proteins and found that the *p*-aminophenylglycosides of the terminal

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non-reducing hexose in appropriate configuration were almost as good inhibitors as the *p*-aminophenylglycosides of the disaccharides of which they represented the terminal non-reducing unit. More recently open chain oligosaccharides of the maltose series were found to inhibit precipitation of antidextran of 1→4 specificity by a dextran with a large proportion of 1→4 linkages while Schardinger dextrins showed no inhibiting power. Maltoheptaose, in which the ability to form a helical structure would interfere with the freedom of the terminal non-reducing group, showed substantially lower inhibiting capacity than oligosaccharides of shorter chain length. Heidelberger, Dische, Neely and Wolfrom[28] studied the cross reaction of lung galactan and tamarind seed polysaccharide with Type XIV antipneumococcal serum and attributed the cross reaction at least in part to the occurrence of multiple terminal non-reducing galactosidic residues.

It should also be pointed out that in contrast to the studies on azo proteins from which it was concluded that charged molecules were important determinants of immunological specificity,[15] the present work clearly shows high degrees of specificity of uncharged molecules as was also reported by previous workers.[27,29] It would thus seem that the combination of antigen and antibody may take place and that sufficient energy for a firm antigen–antibody bond may be provided by hydrogen bonding, van der Waals forces, etc., without any necessity for electrostatic interaction between antigen and antibody. This was also pointed out in the case of polysaccharides of the tubercle bacillus by Heidelberger and Menzel.[30]

From the data presented in Figs. 1 and 2, it is possible to draw some inferences as to the length of the active A and B specific oligosaccharide side chains by comparison of the inhibiting power of these oligosaccharides with the isomaltose series of oligosaccharides used to inhibit dextran–antidextran precipitation.[14] About 0.25 μ mole of isomaltotriose was found capable of giving 50% inhibition of precipitation of antidextran of 1→6 specificity by dextran. Further studies[31] with isomaltohexaose kindly provided by Drs. J. R. Turvey and W. J. Whelan[32] have shown that as little as about 0.07 μ mole of isomaltohexaose was needed for 50% inhibition. In these studies about 3 μ mole of raffinose, stachyose or melibiose was required for 50% inhibition in the B–anti-B system and 1.8 mg. of the oligosaccharide fraction of hog mucin or about 3 μ moles expressed as a trisaccharide gave 58% inhibition of the A–anti-A system. Thus the power of the materials to inhibit blood group A or B activity is only about $^1/_{12}$ to $^1/_{40}$ that of the isomaltose oligosaccharides in their homologous system. If the dimensions of the antibody combining sites on human anti-A and anti-B are similar to those of antidextran and the bond strengths in these two systems are of the same magnitude, one would infer that the active A and B oligosaccharide chains on the blood group substances are probably of the order of tri- to penta- or hexasaccharides.

Such a conclusion represents a substantial simplification of the entire problem of blood group A, B and O specificity since it implies that the bulk of the blood group substance molecule may function as a carrier for multiple specific oligosaccharide side chains which endow it with blood group activity. Indeed the finding that a 1→6-α-galactosido linkage gives substantial inhibition of B–anti-B precipitation also suggests that the numerous extensive cross reactions and species differences among the B substances (as well as among the A substances) may depend upon the widespread occurrence of such linkages. It also points the way both to the elucidation of the structure of these oligosaccharide side chains and to their ultimate synthesis. The former objective readily may be approached by chromatographic fractionation of the dialyzable oligosaccharides obtained from mild acid hydrolysis, determination of the capacity of any materials obtained to inhibit precipitation in the homologous blood group system and selection of the one with structure most complementary to the antibody combining site. The problem of synthesis may also be investigated by the preparation of oligosaccharides of known structure, with increased capacity to inhibit precipitation as a guide in the construction of larger and more active units. Such A and B specific oligosaccharides, if substituted as repeating units on almost any polysaccharide molecule would be expected to convert it to a substance with blood group specificity. Indeed from the potency of melibiose as an inhibitor in the B–anti-B system (Fig. 2) (over 60% inhibition), one would anticipate that the introduction of a number of melibiosyl residues into a polysaccharide would endow it with substantial blood group B activity. Studies along these lines are in progress.

NOTE ADDED IN PROOF.—Watkins and Morgan (*Nature*, **175**, 676 (1955)) have obtained independent evidence for the role of N-acetylgalactosamine and galactose in A and B specifically, respectively, by demonstrating that these sugars specifically inhibited the enzymatic activity of crude extracts of *Trichomonas foetus* on the A and B substances respectively; fucose inhibited the action of the enzyme on O(H) substance.

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