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

Immunochemical Studies on Blood Groups. XXI. Chromatographic Examination of Constituents Split from Blood Group A, B and O(H) Substances and from Type XIV Pneumococcal Polysaccharide by *Clostridium tertium* Enzymes[1]

BY GERALD SCHIFFMAN, CALDERON HOWE[2] AND ELVIN A. KABAT

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Enzymes from two strains of *Clostridium tertium*, Iseki and McClung (1259), act on blood group substances and type XIV pneumococcal polysaccharide to split off about 4 and 12%, respectively, of dialysable material as measured by reducing sugar. These dialysable components were fractionated chromatographically and where possible identified. From the mono- and oligosaccharides split off and from the immunochemical changes caused by these enzymes an attempt was made to correlate structure and specificity. Iseki strain enzymes split off from blood group A substance galactose, N-acetylglucosamine, a trace of N-acetylgalactosamine and a disaccharide of galactose and N-acetylgalactosamine. From O(H) substances, the Iseki enzymes split off galactose and a compound which appears to be a substituted N-acetylhexosamine. The McClung (1259) enzymes split off from human and hog blood group A substances galactose, N-acetylglucosamine and a disaccharide. Only galactose and N-acetylglucosamine were obtained by the action of McClung (1259) enzymes on human B substance. When either enzyme acts on SXIV only galactose is released, amounting to about 18% of the SXIV. Both enzymes can split lactose, galactosyl 1→4 β-N-acetylglucosamine, β-methyl N-acetylglucosaminide and to a small extent galactosyl 1→3-β-N-acetylglucosamine but no α-linked galactose containing compounds. From the specificity of the enzymes, it is concluded that the ability of the blood group substances to cross react with anti-SXIV is in part attributable to terminal non-reducing β-linked galactose probably attached to carbon 4 of N-acetylglucosamine. Although blood group A activity is lost by the action of Iseki enzymes only a trace of free N-acetylgalactosamine was liberated into the dialysate. After having first been digested with McClung (1259) enzymes, blood group A substances can be inactivated by Iseki enzymes without the appearance in the dialysate of any material which could be attributed to the destruction of the specific antigenic site.

In the preceding paper,[3] a summary was given of various studies in which enzymes have been used to explore the relationship between structure and immunological specificity of blood group substances. In addition, Iseki and Masaki[4] have reported that the only chemical changes associated with destruction of blood group O(H) activity by enzymes of *B. fulminans* or of blood group A activity by enzymes of *Cl. tertium* was the liberation of a small quantity of fucose. Reducing sugar, hexosamine and amino acid content remained essentially unchanged. Similar results were obtained by Iseki and Ikeda[5] using human B substance and enzymes from *B. cereus*. As also mentioned in the preceding paper, enzymes from *Trichomonas foetus*, *Lactobacillus bifidus*, *Clostridium welchii* and snail liver apparently degrade blood group substances more extensively and their action results in the release of a variety of compounds.

From studies of inhibition of precipitation[6] and hemagglutination[7,8] the monosaccharides most closely associated with A, B and O(H) blood group activity are N-acetylgalactosamine, galactose and L-fucose, respectively. These sugars are capable also of inhibiting enzymatic destruction of their respective blood group substances.[9,10] This paper describes the results of chromatographic fractionation of the dialyzable products arising from the action of enzyme preparations from the Iseki and McClung strains of *Cl. tertium*[3,11] on blood group A, B and O(H) substances and on type XIV pneumococcal capsular polysaccharide (SXIV). It has been found that both the Iseki and McClung (1259) enzymes possess β-galactosidase activity specific for terminal β-galactosyl residues linked to carbon 4 of glucose or N-acetyl-D-glucosamine and to a small extent to β-galactosyl residues linked to carbon 3 of N-acetylglucosamine and liberate galactose, in addition to other compounds from blood group A, B and O(H) substances but split off only galactose from SXIV. The loss of cross reactivity of blood group substance with type XIV antibody (anti-SXIV) is attributable to the splitting off of terminal β-linked galactose. This interpretation is in agreement with the immunochemical findings of Heidelberger[12] who concluded that the presence of terminal non-reducing galactose units in gums and polysaccharides permitted cross reaction with anti-SXIV. Heidelberger, Barker and Stacey[13] found from infrared studies that the galactose in SXIV was predominantly β-linked. Moreover, Barker, Heidelberger, Stacey and Tipper[14] have isolated from methylated SXIV 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-glucose, 3-O-methyl-D-glucosamine, 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose. From these compounds terminal galactose residues linked to carbon 4 of glucose or of N-acetyl-D-glucosamine as the second residue are definite possibilities as is a terminal galactose linked to carbon 3 of a second galactose. Galactose linked to carbon 3 of N-acetylglucosamine would appear to be ruled out if 3-O-methyl-D-glucosamine accounted for all the amino sugar.[14]

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(2) Markle Scholar in Medical Science.

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Furthermore, qualitative[15] and quantitative[16] studies have shown that β-galactosides and especially galactosyl 1→4β-N-acetyl-D-glucosamine, lactose and galactosyl 1→3β-N-acetyl-D-glucosamine are most effective in inhibiting the precipitation of horse anti-SXIV by intact[16] and partially hydrolyzed[15,16] blood group substances.

Loss of blood group A activity from hog or human A substances treated with Iseki enzymes is accompanied by liberation of a small quantity of N-acetylgalactosamine which is not found when the same materials are treated with McClung (1259) enzyme preparations. In addition to galactose, the Iseki enzymes split off a substituted N-acetylhexosamine from hog O(H) substances. Galactose, N-acetylglucosamine, a trace of N-acetylgalactosamine and a disaccharide of galactose and N-acetylgalactosamine are split from blood group A substance. Since no N-acetylhexosamine is split from SXIV by these enzymes, the N-acetylglucosamine residues in SXIV must be arranged differently from those which are liberated from the blood group substances.

Experimental

Chromatographic Methods.—The Darco–Celite column described by Whistler and Durso[17] was used with modifications. The Darco G-60 was treated with 4–5 volumes of 4 *N* HCl, washed free of chloride and dried *in vacuo* over NaOH and P_2O_5. A column was prepared by mixing equal weights of washed Darco and Celite No. 535, adding 10 volumes of water to make a loose slurry, subjecting the slurry to a vacuum for half an hour with occasional shaking to remove trapped air and pouring the slurry into a glass chromatographic tube containing a layer of Celite over the sintered glass disc. The slurry was allowed to settle and form the column, then gentle positive pressure was applied to the top of the column by blowing. The packed column was attached to an automatic fraction collector. The charge was added and washed into the column with 3 or 4 rinses of water and collection of samples started. Water was used as the developer until the monosaccharides were eluted. A two stage alcohol gradient was then imposed for the rest of the chromatogram. A typical column for the fractionation of a maximum of about 3 mg. of reducing sugar is prepared by mixing 1.5 g. of Darco G-60 and 1.5 g. of Celite 535 which when treated as above forms a column 9 × 160 mm. Ruled chromatographic tubes (9 × 280 mm.) with standard taper (10/18) sintered glass disc bottoms were used.[18] Fractions of 3 ml. were collected at a flow rate of 10 ml./hour. Glucose and galactose were eluted with 20 to 30 ml., and fucose with about 150 to 180 ml. of water. N-Acetylglucosamine and N-acetylgalactosamine did not separate completely from one another; both were eluted at 1 to 2% ethanol. Disaccharides were eluted at 3 to 5% ethanol. Chromatograms were usually complete when the alcohol concentration was about 20%. When a two stage gradient, 0 to 50% ethanol, was used with 500-ml. volumes for each stage, 20 to 25% ethanol concentration was achieved with 1000 to 1200 ml. of effluent. Glucosamine and galactosamine were separated on the Dowex-50 column as described by Gardell.[19] An 8 g. column was needed to effect separation when 0.3 *N* HCl was used for elution. Samples of 0.5 to 1.0 ml. were collected at a flow rate of 1.5 to 3 ml./hour.

Analytical Methods.—Reducing sugar values were measured by a modification of the procedure of Park and Johnson.[20] The reagents used were: (1) **ferricyanide solution**—400 mg. of potassium ferricyanide per liter; (2) **carbonate–cyanide solution**—5.3 g. sodium carbonate, 1.3 g. of potassium cyanide per liter; (3) **ferric–Duponol solution**—1.5 g. of ferric ammonium sulfate, 1.0 g. of Duponol (ME dry) and 2.0 ml. of concentrated sulfuric acid per liter. To each 1.0-ml. sample in a 5-ml. volumetric flask with a ground glass stopper, 1.0 ml. of freshly made mixture of equal volumes of reagents 1 and 2 were added. The contents of the flasks were mixed, tightly stoppered and heated for 20 minutes in a boiling water-bath. After heating the flasks were cooled in tap water for one minute, 2.5 ml. of reagent 3 added and the volume brought to 5.0 ml. with water. The solutions were read in a Beckman spectrophotometer at 6900 Å. after 20 minutes at room temperature. The modifications were introduced (1) to reduce the sample size by decreasing the final volume; (2) to reduce the blank values by reducing the ferricyanide concentration; (3) to increase the sensitivity by increasing the cyanide concentration and the heating period[21]; (4) and to avoid the occasional turbidity which developed when less than 2.0 ml. of sulfuric acid per l. in reagent 3 was used.

Ethanol concentration in the effluent was determined by a spectrophotometric modification of a procedure using Anstie's reagent. To a sample of 0.23 ml. containing 0.1 to 3.0 μl. of ethanol. 2.0 ml. of Anstie's reagent[22] was added, the solution mixed and read at 6050 Å. Carbohydrates in the concentrations encountered did not interfere.

Methylpentose was determined by the method of Dische and Shettles.[23] Galactose was determined by the method of Dische.[24] Amino nitrogen was determined by a ninhydrin procedure similar to that described by Moore and Stein[25] and more recently by Rosen.[26] Immediately before use 0.5 ml. of *M*/100 KCN is added to a solution of 200 mg. of ninhydrin in 20 ml. of methyl Cellosolve. To a 0.1-ml. sample containing 0.05 to 2.0 μg. of amino nitrogen, 0.1 ml. of 2 *M* acetate buffer *p*H 5.5 are added followed by 0.2 ml. of the KCN–ninhydrin solution. The solution is mixed, heated at 100° for 30 minutes and cooled in tap water. One ml. of 50% ethanol is added and after mixing readings are made at 5700 Å. When 1–2 μg. of amino N are present, 2.0 ml. of 50% ethanol is added to give a convenient optical density. The color is stable for several hours.

Hexosamine was determined by a modified method of Elson and Morgan[27] after hydrolysis in 2 *N* HCl for 2 hours at 100° in a tightly stoppered 10-ml. volumetric flask and evaporation to dryness in a vacuum desiccator.[28] N-Acetylhexosamine was determined by the method of Reissig, *et al.*[29] A 0.23-ml. sample containing 2–10 μg. of N-acetylhexosamine is heated at 100° for 3 minutes with 0.05 ml. of 0.8 *M* tetraborate, cooled and 1.5 ml. of Ehrlich reagent added. The color is allowed to develop at 37° for 20 minutes after which it is read at 5440 Å. in a spectrophotometer. By this method N-acetylgalactosamine yields 31% as much color as does an equal quantity of N-acetylglucosamine. Roseman[30] has determined glucosamine and galactosamine in mixtures on this principle. This difference made it possible to distinguish between glucosamine and galactosamine after separation on Dowex-50. The acid eluate was evaporated in a desiccator, the sample dissolved in a known volume of water and aliquots were removed for reducing sugar and amino nitrogen analyses. Another aliquot calculated to contain 2–10 μg. of hexosamine in 0.20 ml. was then converted to the N-acetyl derivative by the procedure of Roseman, adding 0.02 ml. of saturated sodium bicarbonate and 0.02 ml. of ice-cold 5% aqueous acetic anhydride and allowing to stand for 10 minutes at room temperature. The excess acetic anhydride is destroyed by heating at 100° for 3 minutes, 0.050 ml. of 0.8 *M* tetraborate was added and the sample again heated at 100° for exactly 3

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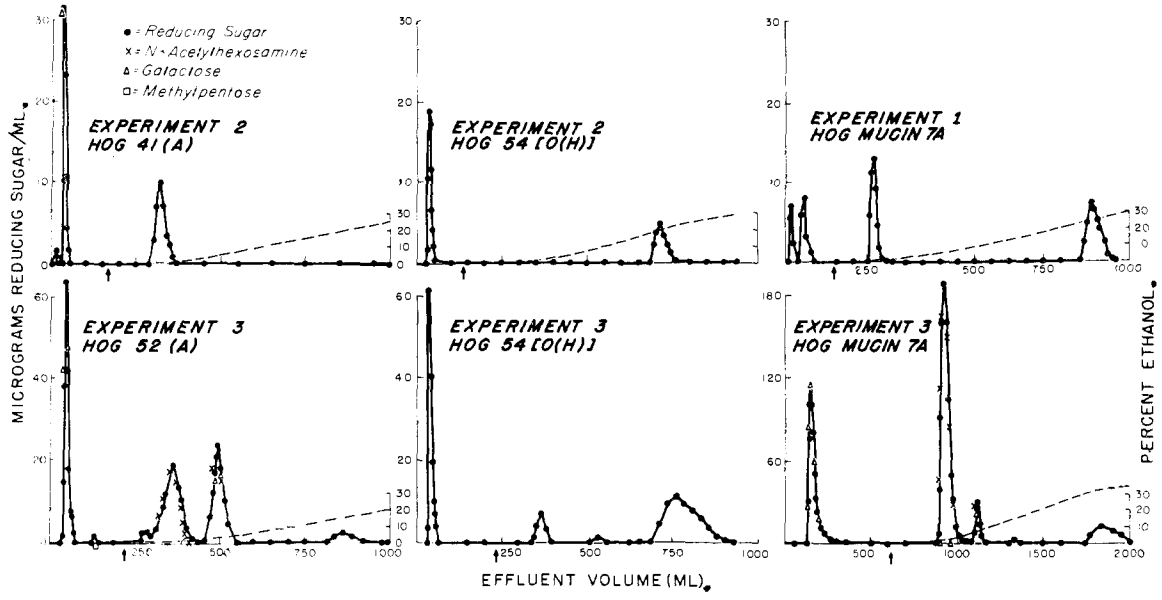

Fig. 1.—Action of Iseki enzymes on hog blood group substances. Each graph represents the chromatographic pattern obtained when dialysates were fractionated on a Darco–Celite column. Except for experiment 3 hog mucin 7A the columns consisted of 1.5 g. of Darco G-60 and 1.5 g. of Celite 535 in which the column consisted of 9 g. of Darco G-60 and 9 g. of Celite. The arrow represents the point at which the two stage ethanol gradient was imposed. The dashed lines represent the ethanol concentration in the effluent. In experiment 3 hog 54 (O(H)) the ethanol concentration was not determined.

minutes and the N-acetylhexosamine color determined as above. The sodium acetate formed did not interfere. Five to six μg. of an unknown hexosamine were sufficient to achieve identification as glucosamine or galactosamine.

N-Acetylglucosamine and N-acetylgalactosamine which do not separate completely from each other on a Darco column were determined by a procedure which again takes advantage of the different color yields of the acetylated hexosamines. When reducing sugar is measured with glucose as a standard and N-acetylhexosamine with N-acetylglucosamine as a standard, then the total reducing sugar value of a solution containing both N-acetylglucosamine and N-acetylgalactosamine is: reducing sugar (glucose equivalent) = 0.80 N-acetylglucosamine + 0.69 N-acetylgalactosamine. Similarly the N-acetylglucosamine equivalent of the mixture is: N-acetylhexosamine (N-acetylglucosamine equivalent) = 1.00 N-acetylglucosamine + 0.31 N-acetylgalactosamine. Solving these equations for N-acetylglucosamine and N-acetylgalactosamine in terms of reducing sugar, R, and N-acetylhexosamine, NA, values one gets

N-acetylgluosamine = 2.28 R − 1.82 NA

N-acetylgalactosamine = 1.59 NA − 0.72 R

where R is reducing sugar value glucose equivalent and NA is the N-acetylhexosamine value measured with N-acetylglucosamine as the reference standard. When the method is used frequently, it has been found convenient to solve the equations graphically. Determination of N-acetylglucosamine and N-acetylgalactosamine by reducing sugar and acetylhexosamine equivalents rather than by hexosamine and acetylhexosamine equivalents has the advantage of requiring only 5–6 μg. of acetylhexosamine whereas the hexosamine determination requires several times this quantity. It is recommended, because of the very small scale, that each laboratory determine their own values for N-acetylglucosamine, N-acetylgalactosamine and reducing sugar equivalents.

The spectrophotometric method of Dixon and Lipkin[31] was used to determine the periodate consumption of oligosaccharides. It was found that the reaction could not be carried out directly in quartz cells. Spectrophotometric determination of the periodate consumed was possible when the reaction was conducted in Pyrex test-tubes away from direct sunlight from which aliquots were removed at intervals and diluted to 1.0 ml. and the optical density measured at 2225 Å. The formaldehyde released by periodate oxidation was measured by the chromotropic acid reaction after destruction of the excess periodate by arsenite as described by Smith and Montgomery.[32]

(31) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

Results

Action of Iseki Enzymes on Blood Group Substances.—Figure 1 shows the fractionation obtained on a Darco–Celite column of dialysable constituents, 3.5 to 5.1% of blood group substances used, liberated by the action of Iseki enzymes on two hog A, two hog O(H) and two hog mucin (A, O(H)) preparations as well as the alcohol gradient during the progress of the columns. In all six graphs the major peak eluted with water was galactose. In five of the six cases it was the only sugar eluted with water. In the sixth case (hog mucin 7A experiment 1) the galactose peak was preceded by a small amount of a reducing sugar component, 69 μg., which gave a hexosamine reaction and an N-acetylhexosamine color after, but not before, acetylation which was 30% that of N-acetylglucosamine. It gave no color test for either methylpentose or galactose. From the position at which galactosamine appears on elution from the column and from the quantitative color reactions, it may be concluded that this compound is galactosamine. It was never encountered again throughout this study and was the only instance in which dialyzed culture supernatant rather than ammonium sulfate precipitated enzymes were used. The major peak which was eluted from each of the columns with water was identified as galactose on the basis of the following evidence: (1) the Dische color reaction for galactose accounted quantitatively for all the reducing sugar within experimental error; (2) absence of hexosamine, N-acetylhexosamine and

(32) F. Smith and R. Montgomery in "Methods of Biochemical Analysis," Vol. 3, Interscience Publishing Co., New York, N. Y., 1956 p. 197.

methylpentose; and (3) the position on the chromatogram, which corresponded to that at which galactose is known to be eluted. The amounts of galactose in the peaks isolated from the column ranged from 0.13 to 4.5 mg. which was 0.5 to 1.3% of the weight of blood group substances used, when corrected for the aliquot used for chromatography. The next major peak appeared only with the two hog A substances and the two mucins and was found only in very small amount with hog 54 O(H) in experiment 2. This peak appeared in the effluent when the ethanol concentration reached 1 to 2%, the position at which known samples of N-acetylglucosamine and N-acetylgalactosamine are eluted. This peak contained no methylpentose or galactose but did give positive N-acetylhexosamine reactions and on hydrolysis yielded hexosamine. Since N-acetylglucosamine and N-acetylgalactosamine do not separate completely from one another in this system, quantitative analyses were performed on individual tubes for both reducing sugar and N-acetylhexosamine when sufficient material was present. As described in the previous section this permitted calculation of the amounts of N-acetylglucosamine and of N-acetylgalactosamine in the sample. The results showed that the peaks emerging at 1 to 2% ethanol were about 99% N-acetylglucosamine. In the two cases in which the analyses were performed, namely, experiment 3 hog 52 (A) and mucin 7A, 25 and 100 μg., respectively, of N-acetylgalactosamine were found in the tailing edge of the 1.1 and 10.0 mg. of N-acetylglucosamine. The existence of 100 μg. of N-acetylgalactosamine in experiment 3 hog mucin 7A was further confirmed by hydrolysis of the pooled tubes from the tailing edge of the peak in 2 *N* HCl for 2 hr. at 100° and chromatography of the free hexosamine bases on a Gardell column of Dowex-50. Elution with 0.3 *N* HCl revealed two peaks in the proper places for glucosamine and galactosamine. These peaks both gave equivalent values when measured by reducing sugar and ninhydrin tests. On acetylation the first peak gave 100% of the color expected for N-acetylglucosamine and the second peak gave 31% of the color of N-acetylglucosamine, as expected for N-acetylgalactosamine. The N-acetylglucosamine in the major portion of all the peaks eluted at 1 to 2% ethanol (Fig. 1) was further identified by its mobility on paper electrophoresis in 0.2 *M* borate buffer *p*H 9.8 at 12 to 13 milliamperes for 4 hr. Under these conditions N-acetylglucosamine, N-acetylgalactosamine and N-acetylmannosamine[33] could be separated readily. The two samples from hog mucin (7A and Fr II in experiment 3 and 7) migrated identically with N-acetylglucosamine. Moreover, the optical rotation of the samples was in the right range for N-acetylglucosamine; accurate measurements could not be obtained with the small amounts of material available.

The next peak was eluted from the Darco–Celite column at 5% ethanol and was present only in experiment 3 with hog 52 (A) and mucin 7A. The amounts isolated from the columns on which 90 and 78% of the total dialysates had been placed were 0.93 mg. from hog 52 (A) and 0.68 mg. from mucin 7A calculated as reducing sugar. This compound contained galactose and N-acetylhexosamine but no methylpentose. On hydrolysis the only hexosamine base present was identified as galactosamine by (1) chromatography on Dowex-50 and acetylation, and (2) by hydrolysis of the disaccharide, acetylation of the free base and paper electrophoresis. Since the original disaccharide reacted with Ehrlich's reagent, it is evident that the N-acetylgalactosamine must be on the reducing end and from the findings of Kuhn, Gauhe and Baer[34] that its hydroxyl group on carbon 4 must be unsubstituted. Since positions 1, 2, 4 and 5 are unavailable for substitution, assuming a pyranose ring, the galactosyl residue could be attached either at position 3 or 6. When the disaccharide was oxidized with sodium periodate at room temperature in an unbuffered solution, one mole of formaldehyde was released, as measured by the chromotropic acid reaction, being formed from the primary unsubstituted hydroxyl on carbon 6 of the reducing residue. This conclusion was substantiated by periodate oxidation under the same conditions, of galactosyl 1 → 3β-N-acetylglucosamine,[35a] galactosyl 1 → 4β-N-acetylglucosamine,[35b] and galactosyl 1 → 6β-N-acetylglucosamine.[35c] Formaldehyde was liberated only from galactosyl 1 → 3β-N-acetylglucosamine and galactosyl 1 → 4β-N-acetylglucosamine but not from galactosyl 1 → 6β-N-acetylglucosamine as would be expected theoretically. Thus the compound could not be 1 → 6 linked. The liberation of formaldehyde also excludes a 1 → 5 linkage with a furanosidic reducing residue. The rate of formaldehyde formation on periodate oxidation showed that one mole was released in 3 hours when the unknown disaccharide was oxidized whereas 20 hours were required for the release of 0.5 mole from galactosyl 1 → 3β-N-acetylglucosamine and 60 hours were required for the release of 0.5 mole from the galactosyl 1 → 4β-N-acetylglucosamine. The liberation of one mole of formaldehyde from galactosyl 1 → 3β-N-acetylglucosamine required 120 hours and from galactosyl 1 → 4β-N-acetylglucosamine 145 hours. After borohydride reduction, which destroys the ring structure and produces a vicinal glycol on carbons 5 and 6 of the hexosamine residue, formaldehyde production by periodate was complete within one hour at room temperature from galactosyl 1 → 3β-N-acetylglucosamine and galactosyl 1 → 4β-N-acetylglucosamine as well as from the isolated disaccharide. The rate of periodate uptake at room temperature was followed spectrophotometrically over a period of 22 hours. Galactosyl 1 → 4β-N-acetylglucosamine consumed 1.6 moles in one hour, 2.1 moles in 4.5 hours and 3.5 moles in 22 hours. Galactosyl 1 → 3β-N-acetylglucosamine used 2.6 moles of periodate in one hour and 2.8 moles in 22 hours. The disaccharide isolated from hog 52 (A) and hog mucin 7A consumed periodate at a rate equal, within experimental error, to that of galactosyl 1 → 3β-N-acetylglucosamine. The isolated di-

(33) (a) A sample of mannosamine was kindly supplied by Dr. S. Roseman. (b) D. G. Comb and S. Roseman, THIS JOURNAL, **80**, 497 (1958).

(34) R. Kuhn, A. Gauhe and H. H. Baer, *Chem. Ber.*, **87**, 1138 (1954).

(35) Kindly supplied by (a) Dr. F. Zilliken, (b) Dr. R. M. Tomarelli and (c) Prof. R. Kuhn.

saccharide is therefore tentatively thought to be a galactosyl 1 → 3N-acetylgalactosamine.

The last compound was eluted at 15–20% ethanol and was found in dialysates from experiment 1 with hog mucin 7A (0.42 mg. as reducing sugar), experiment 2 hog 54 O(H) (0.84 mg. as reducing sugar) and mucin 7A (1.76 mg. as reducing sugar). Aliquots of dialysate used in these four columns were 40, 50, 90 and 78%, respectively. Only a small amount (0.12 mg. as reducing sugar) was found in experiment 3 hog 52 (A) in which 90% of the total dialysate was used and none in experiment 2 hog 41 (A) in which 50% of total dialysate was used. This compound gave an N-acetylhexosamine reaction, and on hydrolysis a hexosamine reaction but contained neither methylpentose nor galactose. The hexosamine value was equivalent to the reducing sugar value unhydrolyzed and did not increase on hydrolysis in 2 *N* HCl for 2 hours or 6 *N* HCl for 24 hours. The ratio of N-acetylhexosamine to reducing sugar was 1.65–1.75 as compared to 1.25 for N-acetylglucosamine and 0.44 for N-acetylgalactosamine. The colored products formed in both the N-acetylhexosamine and hexosamine reactions are similar to those given by N-acetylglucosamine and glucosamine with maxima at 5440 and 5880 Å. in the former and 5400 Å. in the latter assay. Unlike the sialic acids, the compound gave a color reaction with Ehrlich's reagent in the cold without prior alkaline treatment. On elution from a Darco–Celite column and in its N-acetylhexosamine to reducing sugar ratio this compound resembled N-acetylmuramic acid (3-O-α-carbethoxy-N-acetylglucosamine), which is eluted at 12–15% ethanol and has an N-acetylhexosamine to reducing sugar ratio of 1.8. It was definitely not N-acetylmuramic acid, however, because on hydrolysis in 2 *N* HCl for 2 hours at 100° glucosamine was found and identified by chromatography on Dowex-50 and on subsequent acetylation. N-Acetylglucosamine as determined by the amount of N-acetylhexosamine color per unit weight was obtained. Under these conditions N-acetylmuramic acid yields muramic acid since the 3-O-carbethoxy ether linkage is stable to these hydrolytic conditions. Moreover, muramic acid is not eluted from the Dowex-50 column with 0.3 *N* HCl but requires 0.8*N* HCl as shown by Park.[36] The chromogen formed by muramic acid before and after hydrolysis in 2 *N* HCl for 2 hours at 100° in the Elson–Morgan reaction has an absorption peak at 5100 Å. which is significantly different from 5400 Å., the absorption maximum of glucosamine and of the base liberated on acid hydrolysis.

On paper electrophoresis[37a] at *p*H 9.8 in borate buffer for 4 hours at 12 milliamperes and development as in reported,[37b] N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, the isolated N-acetylhexosamine and N-acetylmuramic acid migrate toward the cathode 1 to 2, 2 to 5, 8, 8.5 and 8.5 cm. from the origin, respectively. After acid hydrolysis in 2 *N* HCl at 100° for 2 hours and reacetylation each of the above compounds migrated as they did before hydrolysis with the exception of the N-acetylhexosamine liberated by enzyme which migrated indistinguishably from N-acetylglucosamine.

When the isolated N-acetylhexosamine compound was subjected to de-O-acetylation conditions by two different methods, barium methylate in methanol or aqueous sodium hydroxide[38] at 0°, the ability to react with Ehrlich's reagent in the cold was somewhat enhanced rather than lost. Thus the substitution on the glucosamine-like base does not appear to be a simple alkali sensitive ester.

Action of McClung (1259) Enzymes on Blood Group Substances.—Figure 2 shows the chromatographic patterns obtained by the action of McClung strain enzymes on human and hog blood group A, human B and hog O(H) substances. All the chromatograms show galactose as the only sugar eluted with water. In all four preparations, N-acetylhexosamine was eluted at 1 to 2% ethanol, and except for blood group B substance, a third peak was eluted at 5% ethanol.

Difficulty was encountered in the chromatography of the dialysates from hog O(H) and human B substances treated with McClung enzymes in that none of the sugars was absorbed on the Darco–Celite column but passed through entirely in the aqueous eluate as determined by analytical recovery of the total reducing sugar and N-acetylhexosamine originally placed on the column. On concentrating and rechromatographing on a fresh column the chromatograms in Fig. 2 were obtained. The failure of the material to be absorbed on the first column is thought to be due to the presence of amino acids in these dialysates. This difficulty was not encountered in any of the other chromatograms.

The peak eluted by water was identified as galactose by the criteria listed for the Iseki enzyme dialysates. The galactose peak in the hog O(H) dialysate was small, 96 μg. from 75% of the total dialysate. The N-acetylhexosamine peaks from the human A cyst and hog 58 (A), experiment 7, were completely hydrolyzed and chromatographed on a Dowex-50 column. This material was only glucosamine; no evidence of galactosamine was obtained. N-Acetylglucosamine in these samples totalled 1.25 mg. and 0.74 mg., respectively; 80% of the total dialysates were placed on the columns. The absence of N-acetylgalactosamine in this peak was further confirmed on a chromatogram from hog mucin Fr 2 (not shown in graph) from which 5.4 mg. of N-acetylglucosamine was obtained. No evidence for N-acetylgalactosamine in the tailing edge of the peak was found by direct determination in individual tubes of reducing sugar to N-acetylhexosamine ratios. When the tailing edge was pooled, hydrolyzed and chromatographed on Dowex-50, a trace, 10 μg. or less, of a substance which could originally have been N-acetylgalactosamine was found.

The component which appeared in the eluate at 5% ethanol contained galactose and N-acetylhexosamine. Since the ratio of galactose to hexosamine was greater than unity (1.6–2.6) the presence of more than one compound is suspected. The total

(36) J. T. Park—abstracts of 130th Meeting of Division of Carbohydrate Chemistry of the American Chemical Society, Atlantic City, N. J., September 1956, page 18D.

(37) (a) A. B. Foster, *J. Chem. Soc.*, 982 (1953); (b) C. E. Cardini and L. F. Leloir, *J. Biol. Chem.*, **225**, 317 (1957).

(38) "Polarimetry, Saccharimetry and the Sugars," Circular 440, National Bureau of Standards, 1942, page 493.

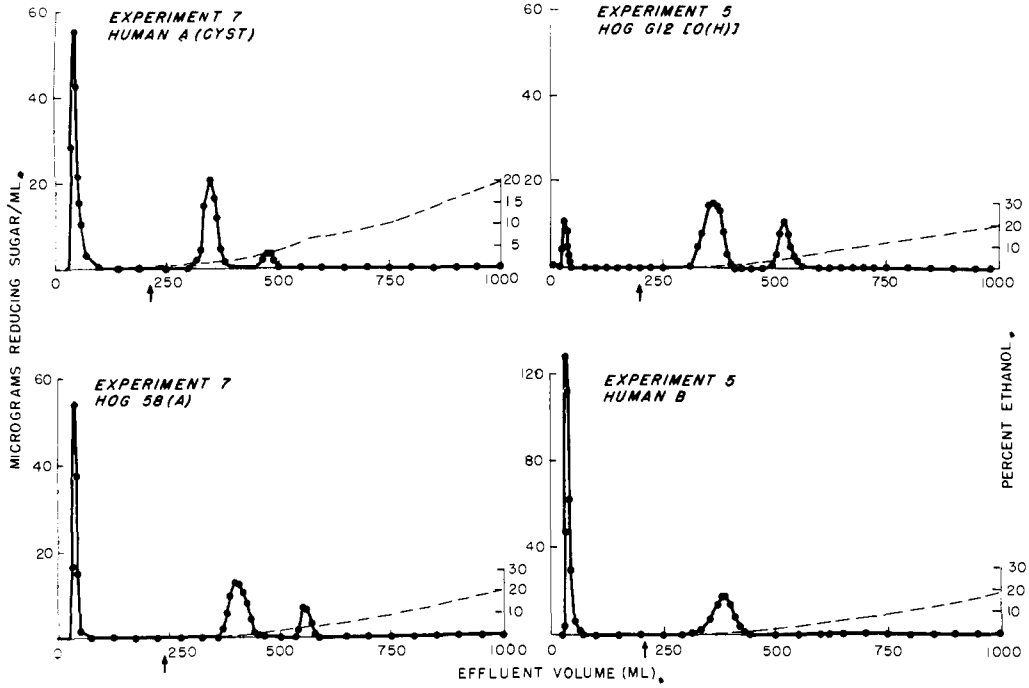

Fig. 2.—Action of McClung (1259) enzymes on hog and human blood group substances. Chromatographic patterns obtained when dialysates were fractionated on columns composed of 1.5 g. of Darco G-60 and 1.5 g. of Celite 535. Ethanol gradient imposed at position designated by the arrow.

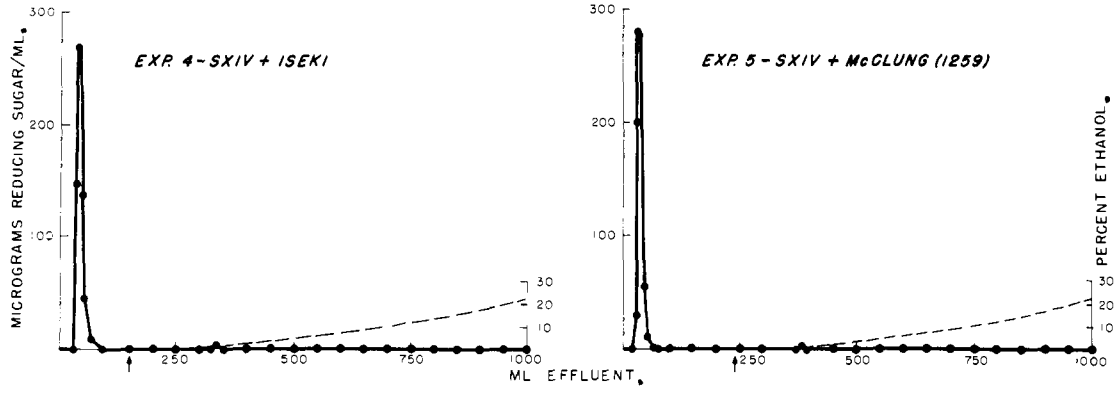

Fig. 3.—Action of Iseki and McClung (1259) strain enzymes on type XIV pneumococcal polysaccharide. Columns consisted of 1.5 g. of Darco G-60 and 1.5 g. of Celite 535. Ethanol gradient imposed at position designated by arrow.

amount of reducing sugar in these peaks amounted to 0.26 and 0.13 mg. from hog 58 (A) and cyst A, respectively, using 80% of the total dialysate for chromatography. The hexosamine base was identified as galactosamine by hydrolysis, acetylation and determination of the N-acetylhexosamine to hexosamine ratio.

Action of *Clostridium tertium* Enzymes on Type XIV Pneumococcal Polysaccharide.—Figure 3 shows the chromatographic patterns obtained when SXIV was treated with Iseki or McClung (1259) enzymes. Only galactose was split off. The galactose peak recovered amounted to 2.7 and 2.9 mg., respectively, as reducing sugar in the 87% aliquots used for chromatography. This reducing sugar represents 11 and 12% by weight of the SXIV. By direct galactose determination on the total dialysate prior to placing on the column, 17 and 19% of the SXIV had been split off by the enzymes. There is no evidence for the presence of any other sugar in the dialysates (Fig. 3).

The galactose liberated from the pneumococcal polysaccharide was probably β-linked as judged from the specificity of the enzyme preparations. Incubation of various substrates with Iseki enzyme preparations at 37° in $M/30$ phosphate buffer at pH 7.4 showed that lactose, galactosyl 1 → 4β-N-acetylglucosamine and β-methyl-N-acetylglucosaminide were split, while cellobiose, melibiose, α-methyl-N-acetylglucosaminide, α-methyl-N-acetylgalactosaminide[39] α-ethyl-N-acetylgalactosaminide,[39] β-ethyl-N-acetylgalactosaminide[39] and galactosyl 1 → 6 β-N-acetylglucosamine were not split. McClung (1259) preparations had a similar specificity with respect to these substrates. On incu-

(39) Kindly supplied by Dr. R. W. Jeanloz.

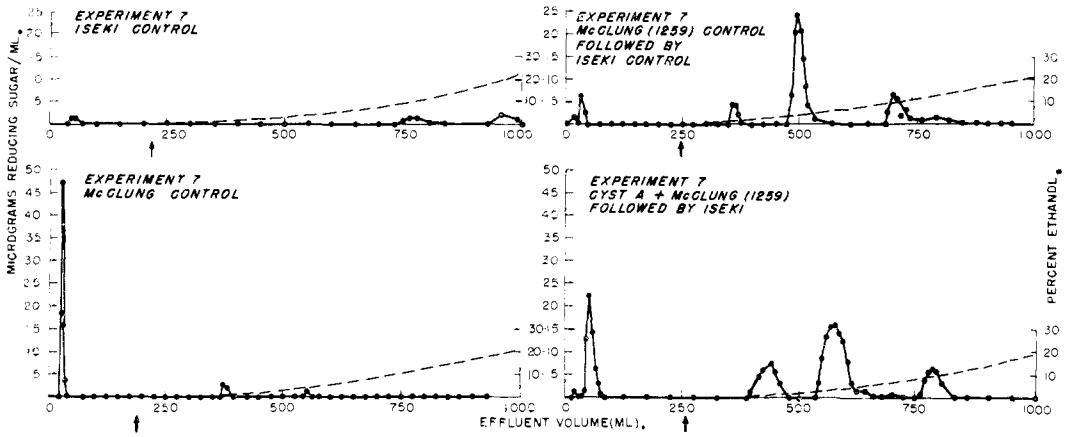

Fig. 4.—Enzyme controls and effects of treatment with Iseki enzymes of human A cyst substances which had first been digested with McClung (1259) enzymes. Column consisted of 1.5 g. of Darco G-60 and 1.5 g. of Celite 535. Ethanol gradient imposed at position designated by arrow.

bation of 488 μg. of galactosyl 1 → 3β-N-acetylglucosamine with 200 μg. of Iseki strain enzyme preparation in *M*/15 phosphate buffer at *p*H 7.4 in 0.5 ml. of final volume at 37° only 9 and 4% increase in reducing sugar was observed at 20 and 68 hours, respectively. Under the same conditions a 58 and 71% increase occurred when galactosyl 1 → 4β-N-acetylglucosamine was the substrate. The McClung (1259) enzymes caused an increase of 15 and 21% at these times with galactosyl 1 → 3β-N-acetylglucosamine as compared with a 59 and 59% increase with galactosyl 1 → 4 β-N-acetylglucosamine. When four times as much Iseki enzymes were used with the same amount of substrates, an increase of 16% in reducing sugar was seen in 2 hours with galactosyl 1 → 3β-N-acetylglucosamine. In 18, 26 and 72 hours the increase over the zero point was 16, 19 and 14%, respectively. With galactosyl 1 → 4β-N-acetylglucosamine increases of 59, 77, 68 and 62% occurred in these time periods. With a similar increase in the amount of the McClung (1259) enzymes, values of 16, 24, 21 and 14% at 2, 18, 26 and 72 hours, respectively, were found with galactosyl 1 → 3β-N-acetylglucosamine. The corresponding increases with galactosyl 1 → 4β-N-acetylglucosamine were 54, 60, 58 and 59%. Controls using heat inactivated enzymes showed changes of about ±5% of the initial values. Thus there appears to be some splitting of a 1 → 3β-galactoside linkage to N-acetylglucosamine with high concentrations of either enzymes; at the lower level of enzyme used (substrate to enzyme ratio of 2.5) the McClung (1259) preparations are capable of splitting this linkage to a small extent whereas the Iseki preparations do not appear to cause significant splitting.

Iseki and McClung Enzyme Controls.—In Fig. 4 it can be seen that no appreciable quantity of reducing sugar was found in the dialysate when 86 mg. of Iseki enzymes was incubated in the absence of substrate. This amount of enzyme was 2–4 times that used in experiments 1 to 7 with the exception of experiment 3 in which the same amount was used with 580 mg. of hog mucin 7A. When 83 mg. of enzyme preparation from the McClung (1259) strain was incubated in the absence of substrate, only one component was found, amounting to 116 μg. of reducing sugar. This component did not contain methylpentose, hexosamine or N-acetylhexosamine and only 26% of its reducing sugar value could be accounted for as galactose. Since only a fraction of this amount of enzyme was used in experiments shown in Figs. 2 and 3, this component contributed a negligible blank.

Mixed Enzyme Control.—When 83 mg. of Iseki and 83 mg. of McClung (1259) enzymes were allowed to digest each other, the dialysates contained several peaks, as can be seen in Fig. 4. The component which was eluted with water amounted to only 39 μg. It did not contain galactose, methylpentose, hexosamine or N-acetylhexosamine. The peak which was eluted at 1 to 2% ethanol, 69 μg., contained N-acetylhexosamine, no galactose or methylpentose. From the ratio of N-acetylhexosamine to reducing sugar, 1.1, the component appears to be N-acetylglucosamine. The compounds which were eluted at 5 and 10% ethanol amounted to 545 and 392 μg.. respectively. They contained the equivalent of one galactose per reducing end group, no N-acetylhexosamine or methylpentose and only a trace of hexosamine. Their structures are not known but probably arise from the carbohydrate constituents shown to be present in the enzyme preparations.[3] This experiment serves as control for the following experiment.

Action of Iseki Enzymes on Cyst A Substance after Previous Digestion by McClung (1259) Enzymes.—Since the action of McClung (1259) enzymes on blood group A substances resulted in the loss of reactivity with anti-SXIV and the concomitant liberation of galactose, N-acetylglucosamine and a disaccharide component, but no loss in blood group A activity, it was thought that digestion with Iseki enzymes of blood group A substance from which the mono and oligosaccharides liberated by McClung (1259) enzymes had first been removed might yield directly oligosaccharides or monosaccharides associated with loss of A activity. Cyst A substance, 85 mg., was digested by 28 mg. of McClung (1259) enzymes, dialyzed and the nondialysable residue treated with 28 mg. of Iseki enzymes. The dialysable components liberated by

the McClung (1259) enzymes are shown in Fig. 2 and have been described. The dialysable components from an aliquot representing 80% of the total material released by the Iseki enzymes on the McClung (1259) treated residue are shown in the lower right chromatogram of Fig. 4. Since the amount of enzymes employed in this experiment is one third that of the control, the quantity of reducing sugar measured was multiplied by three and then plotted on the same scale as the control chromatogram so that the components could be compared visually. The component eluted with water had the same analytical characteristics as that described by the component eluted with water from the McClung (1259) enzymes alone, *i.e.*, no methylpentose or N-acetylhexosamine, a trace of hexosamine and about 35% of the reducing sugar as galactose. This component amounted to 121 μg. of reducing sugar. The next component, 131 μg. of reducing sugar, was eluted at 1 and 2% ethanol and appears to be N-acetylglucosamine since it did not contain methylpentose or galactose and has a reducing sugar to N-acetylhexosamine ratio of 1.2. The next two components, 328 and 113 μg. of reducing sugar, were eluted at 5 and 10% ethanol and had the same analytical characteristics as the two components found in the control chromatograms at 5 and 10% ethanol. These components are assumed to arise from the enzyme preparations and not from the blood group substance.

Although the blood group activity was lost, no evidence of dialysable materials attributable to the action of Iseki enzymes on the blood group substance which had first been digested by McClung (1259) enzymes could be obtained.

Discussion

The findings in this and the previous paper permit certain inferences with respect to the structural units involved in some of the immunological specificities of the blood group A, B and O(H) substances and also contribute in part information on the structure of the type XIV pneumococcal polysaccharide. Since in all instances the McClung (1259) enzymes destroyed type XIV cross reactivity without affecting blood group A, B or O(H) specificity, it is evident that the structures on the intact blood group substances responsible for cross reactivity and for blood group specificity are different. This is substantiated with respect to the B and O(H) substances by the similar findings with the Iseki strain enzymes. This also further clarifies the earlier observation that hog blood group A substances of identical blood group activity could vary widely in their cross reactivity with type XIV antibody [40] The present studies add more evidence for the inference that terminal non-reducing galactosyl residues are responsible for the cross reaction of blood group substances with type XIV antipneumococcal serum in that galactose is the only monosaccharide split off from all three blood group substances (A, B and O(H)) by both enzymes, although the cross reactivity of each of these substances with type XIV antibody was almost completely destroyed. This is consistent with the action of Iseki and McClung (1259) enzymes on SXIV itself from which only galactose was split off, and is in agreement with the finding of terminal galactosyl residues which could be linked 1 → 4 to glucose or N-acetylglucosamine in SXIV by methylation studies.[14,15] This agrees with Heidelberger's[12] assumption that end groups of galactose are important in cross reactivity but renders superfluous the second of his two possibilities, namely, that 1,3 and/or 1,6 and/or 1,3,6 linked galactose residues in the interior of the chains might be partially or completely responsible for cross reactivity of the original blood group substances with type XIV antiserum.[12] If this were so, one would expect substantial residual cross reactivity after enzyme treatment since only a very small amount of material, ranging from 3.5 to 5.1% as reducing sugar and only 0.5 to 3.5% as galactose, is split off from the blood group substances by these enzymes. It is also highly probable, from the known capacity of the enzyme to split galactose linked 1 → 4β to glucose or to N-acetylglucosamine and 1 → 3β to N-acetylglucosamine that a terminal galactose linked 1 → 4β or 1 → 3β to N-acetylglucosamine occurs in the blood group substances; these substances do not contain glucose. This would be in accord with the isolation of galactosyl 1 → 4β-N-acetylglucosamine[41-44] and galactosyl 1 → 3β-N-acetylglucosamine[44] from blood group substances.

Heidelberger suggested the second alternative which attributes at least part of the cross reactivity of the blood group substances and other plant gums with anti-SXIV to galactose residues in the interior of chains because certain of the plant gums contained small numbers of terminal galactosyl residues but showed considerable cross reaction.[12] Another alternative is possible and must be considered, namely, that the capacity of a terminal galactosyl residue to cross react is influenced by the site to which it is attached on a polysaccharide.[45] There is extensive support for such a concept from the oligosaccharide inhibition studies in the dextran antidextran system[46] and in the cross reaction of dextrans with Type II antipneumococcal serum.[47] On this basis all of the variations in cross reactivity of the plant gums with type XIV antibody would be explainable by differences in the attachment of the terminal galactosyl residues.[45] This necessarily implicates at least the subterminal units as influencing in part the degree of cross reactivity.[15,16] This concept is in general agreement with other studies indicating that an antigenic group extends several sugar units (up to six)[46] in from a non-reducing end group. Thus a small number of short chains with a terminal non-reducing galactose and with the next one or more residues with the appropriate structure and configuration (α or β) might produce greater

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(41) Z. Yosizawa, *Tôhoku J. Exptl. Med.*, **51**, 51 (1949); **52**, 111 (1950).

(42) R. M. Tomarelli, J. B. Hassinen, E. R. Eckhardt, R. H. Clark and F. W. Bernhart, *Arch. Biochem. and Biophys.*, **48**, 225 (1954).

(43) R. Kuhn and W. Kirchenlohr, *Chem. Ber.*, **87**, 560 (1954).

(44) R. H. Côté and W. T. J. Morgan, *Nature*, **178**, 1171 (1956).

(45) M. Heidelberger, S. A. Barker and B. Björklund, THIS JOURNAL, **80**, 113 (1958).

(46) E. A. Kabat, *J. Immunol.*, **77**, 377 (1956).

(47) J. W. Goodman and E. A. Kabat, *Feaeration Proc.*, **16**, 415 (1957).

cross reactivity than would a larger number of less specific non-reducing galactosyl residues and thus account for the cross reactions of gums with few galactosyl end groups. Whether a cross reaction or even an homologous reaction between polysaccharides and antibodies can take place with groupings distantly (more than six residues) removed from the ends of chains and without the participation of the non-reducing end groups remains to be shown unequivocally.

The absence of other constituents in the dialysates of the enzyme treated SXIV and the presence of N-acetylglucosamine in the dialysates of the enzyme treated blood group substances indicates that the N-acetylglucosamine of the SXIV is linked differently from that portion of the N-acetylglucosamine of the blood group substances susceptible to enzymic action. The nature of the N-acetylglucosamine linkage in the blood group substances which is susceptible to these enzymes is probably a β-glucosaminidic linkage since the enzymes split β-methyl-N-acetylglucosaminide. It is probably not a branch point since none is liberated by these enzymes from SXIV which presumably[14] has its glucosamine linked 1,4,6. From the finding that it appears in substantial quantities as free N-acetylglucosamine, it is probably either a terminal non-reducing residue or a subterminal residue to which a β-linked galactose is attached probably on carbon four but possibly on carbon 3. In the latter instance the splitting of the β-galactosyl 1 $\rightarrow$ 4 or possibly a 1 $\rightarrow$ 3 bond would render it a terminal non-reducing residue susceptible to the β-glucosaminidase activity of the enzymes.

The reduced effectiveness of the enzyme treated SXIV from which only galactose had been split in precipitating anti-SXIV as compared with the original strongly supports the concept that terminal β-linked galactose is important in the specificity of SXIV. This galactose probably is linked $\beta 1 \rightarrow 4$ to glucose or to N-acetylglucosamine since the enzyme acted most rapidly on compounds with $\beta 1 \rightarrow 4$ linkages and this would agree well with the methylation studies.[14] The enzyme data, however, do not exclude the possibility of $\beta 1 \rightarrow 3$ linkages to N-acetylglucosamine since slight splitting of this compound was also observed. Also the finding that complete splitting of galactosyl 1 $\rightarrow$ 4β-N-acetylglucosamine was not obtained and that only slight splitting of galactosyl 1 $\rightarrow$ 3β-N-acetylglucosamine occurred under the conditions used makes it possible that not all of the terminal galactosyl residues were removed from SXIV by the enzymes despite the finding that the galactose split by the Iseki and McClung enzymes amounted to 17 and 19% of the weight of SXIV used.

A definite difference[3] was found between the SXIV preparations treated with Iseki and those treated with McClung enzyme. Those treated with the former precipitated 82 and 87% of the total anti-SXIV while those treated with the latter precipitated only 68 and 61% of the total anti-SXIV. Since the McClung enzyme split the galactosyl 1 $\rightarrow$ 3β-N-acetylglucosamine more rapidly at lower concentrations than did the Iseki enzymes, the reduced precipitating capacity of SXIV treated with McClung enzyme might be due to it splitting more 1 $\rightarrow$ 3β linked galactoses than did the Iseki enzymes. It is of interest that somewhat more galactose was liberated by the McClung (19%) than by the Iseki (17%) enzymes. Heidelberger, Barker and Björklund[45] have reported that periodate oxidized SXIV in which terminal non-reducing galactosyl residues have been destroyed precipitates only 42% of the total anti-SXIV and methylation studies have given a value of 28% for terminal non-reducing galactosyl residues. It thus appears that the enzyme treated samples of SXIV all of which have substantially greater reactivity with anti-SXIV may still contain terminal galactosyl residues which were not split by the enzymes.

With respect to blood group A specificity, prior data indicate that a terminal N-acetylgalactosamine is involved[6,9,10] and the findings of Côté and Morgan[44] suggest that this is linked α to galactose probably 1 $\rightarrow$ 3. The present studies show that very small quantities of N-acetylgalactosamine are obtained by the action of Iseki enzymes but not by the action of McClung enzymes on A containing materials. The quantities of N-acetylgalactosamine are so small, however, that it does not appear likely that they could account for the loss of blood group activity. Thus from 580 mg. of hog mucin about $^2/_3$ of which represented blood group A substance, only 100 μg. of N-acetylgalactosamine was obtained. Removal of other materials by McClung enzyme and treatment of the non-dialysable residue with Iseki enzyme failed to yield larger amounts of N-acetylgalactosamine. While no definitive explanation can be given to account for the loss of A activity, the possibility must be considered that it results either from the masking of A specific groupings by transglycosylation of one or more irrelevant sugar units over the determinant groups or alternately by transglycosylation of terminal N-acetylgalactosamine residues to some other portion of the blood group molecule and in a position in which they could not function as determinants of blood group specificity. If the latter possibility is correct the small amount of N-acetylgalactosamine might be an indication of incompleteness in the transglycosylation. The finding of the disaccharide of galactose linked 1 $\rightarrow$ 3 to a reducing N-acetylgalactosamine could accord with the former interpretation that a galactosyl residue was transferred on to the terminal N-acetylgalactosamine and that the disaccharide was then split off. There is no conclusive evidence for these inferences, however, and the disaccharide could equally well have been a constituent of the intact blood group substance. These considerations can only be resolved by further investigation.

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