[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL, NEW YORK, N. Y., AND THE MASSACHUSETTS GENERAL HOSPITAL, BOSTON, MASSACHUSETTS]

Immunochemical Studies on Blood Groups. XXVI. The Isolation of Oligosaccharides from Human Ovarian Cyst Blood Group A Substance Including two Disaccharides and a Trisaccharide Involved in the Specificity of the Blood Group A Antigenic Determinant[1]

BY GERALD SCHIFFMAN, ELVIN A. KABAT AND SIDNEY LESKOWITZ

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Blood group A substance derived from human ovarian cyst fluid has been sequentially hydrolyzed under mild conditions (*p*H 1.9 to 1.6 for 2 hr. at 100°). The dialyzable components were fractionated on charcoal, paper and glass fiber sheets to yield three disaccharides and a trisaccharide. By analysis before and after reduction with sodium borohydride, periodate oxidation and optical rotation these compounds were shown to be: (1) α-N-acetylgalactosaminoyl-(1 → 3) galactose, (2) β-galactosyl-(1 → 3)-N-acetylglucosamine, (3) β-galactosyl-(1 → 4)-N-acetylglucosamine and (4) α-N-acetylgalactosaminoyl-(1 → 3)-β-galactosyl-(1 → 3)-N-acetylglucosamine. The trisaccharide was shown to be more active in inhibition of A-anti-A precipitation than the most active disaccharide previously reported and is probably the first three units of the blood group A antigenic determinant.

Earlier studies have shown that of the four monosaccharide units found in blood group A substance, only N-acetylgalactosamine was capable of inhibiting the hemagglutination of A red cells by anti-A,[2] the precipitation of anti-A by A substance[3] and the enzymatic inactivation[4] of blood group A substance.[5] From these observations it was inferred that N-acetylgalactosamine was the terminal non-reducing unit in an A specific oligosaccharide determinant. The isolation of α-N-acetylgalactosaminoyl-(1 → 3)-galactose from a partial acid hydrolysate (0.1 *N* HCl for 3 hr. or 1 *N* HCl for 0.5 hr. at 100°) of blood group A substance[6] and the report[7] that it specifically inhibited hemagglutination of A erythrocytes by anti-A at a much lower concentration than does α-methyl-N-acetylgalactosaminide strongly suggests that this compound represents the first two sugar units from the non-reducing end of the A specific determinant.

Earlier studies from this Laboratory have shown that milder hydrolytic conditions (*p*H 1.5 to 2.0 for 2 hr. at 100°[8]) cleave active groupings from blood group A and B substances leaving a non-dialyzable residue, P1. The mildness of the hydrolytic conditions can be inferred from these observed properties of the P1 fraction: (1) non-dialyzability and alcohol precipitability, (2) increased cross-reactivity with horse type XIV anti-pneumoccocal sera as compared to the original blood group substance, (3) ability to produce circulating precipitins in humans following immunization.[9] These mild conditions were shown to liberate oligosaccharides from blood group A substance capable of specifically inhibiting A-anti-A precipitation.[3] Accordingly, it was thought that further study of the oligosaccharides might yield compounds of two types: (1) active disaccharides different from the one isolated by Côté and Morgan[6] if blood group A specificity involves more than a single antigenic determinant, since subgroups of A are well known, and (2) more active oligosaccharides of longer chain length. This study has resulted in the isolation of three disaccharides and a trisaccharide. The trisaccharide and one of the disaccharides are active in inhibiting A-anti-A precipitation. The four compounds were studied and characterized as to structure.

Experimental

Materials and Methods.—The blood group A substance, McDon, was prepared from the pseudomucinous ovarian cyst fluid[10] of an individual of blood group A. Ethanol was added to 50% and the precipitate obtained was repeatedly digested[5,11] with pepsin at *p*H 2.3. After dialysis and reisolation from 50% ethanol, the dried preparation was extracted with phenol,[12] and ethanol was added to a final concentration of 15% which precipitated the active substance. Twenty grams of this material in 600 ml. was hydrolyzed at *p*H 1.9 for 2 hr. at 100° and dialyzed repeatedly against distilled water. The *p*H of the non-dialyzable solution was adjusted to 1.75 and the material again hydrolyzed and dialyzed. The procedure was carried out a third time at *p*H 1.6. After the three treatments, 10.5 g. of non-dialyzable residue P1 were recovered. Separation of components of the dialysates was achieved initially by gradient elution with ethanol on Darco–Celite[13] as previously described.[14,15] Pooled fractions selected for further purification were chromatographed on washed Whatman 3 MM paper, 50 to 100 mg. per 6" × 17" sheet, and developed with 1-propanol–ethyl acetate–water (7:1:2).[16] After developing small guide strips with alkaline silver nitrate[17,18] to locate

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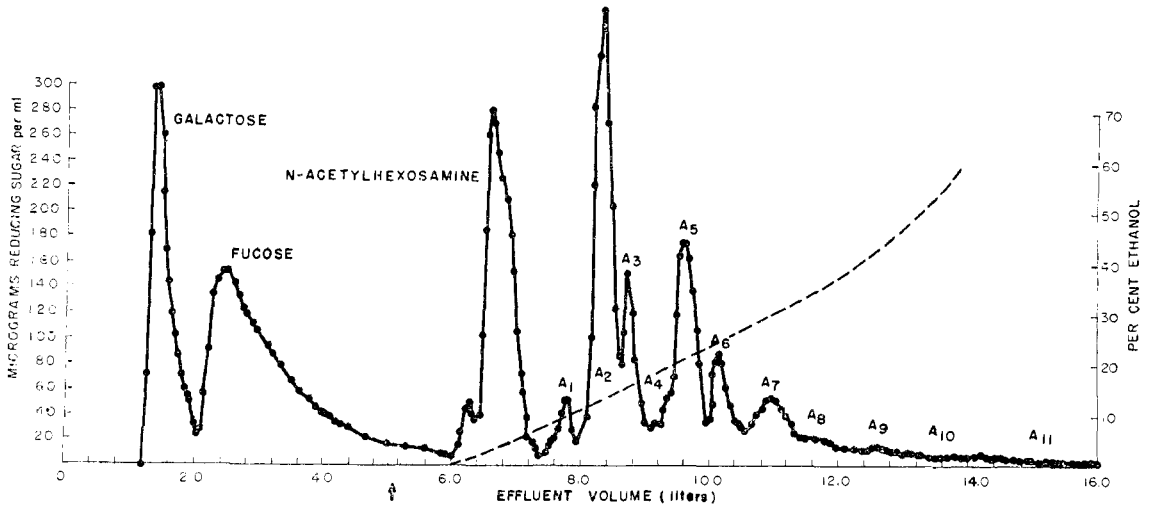

Fig. 1.—Chromatography of 2.46 g. of dialysate on 100 g. Darco–100 g. Celite. The arrow indicates where the two stage gradient was begun; the dashed line represents the ethanol concentration in the effluent.

the compounds, they were eluted with water onto a small quantity of Darco–Celite in a chromatographic column. After copious washing with water, the oligosaccharides bound to the carbon were eluted with 25% ethanol. Two to 3 ml. fractions were collected and the tubes analyzed for reducing sugar.[19,14] The compounds usually are eluted in two to four tubes which are pooled and lyophilized. Further purification was achieved by chromatography on sheets of glass fiber.[20] H. Reeve Angel glass fiber sheets grade 934-AH were impregnated with 0.1 *M* phosphate (*p*H 5.0) and air dried as described.[21] The solvent system used was 1-propanol–ethyl acetate–water (7:1:2).[16] Substantial differences in chromatographic behavior are observed on glass fiber sheets as compared with Whatman paper using a given solvent. Five to 10 mg. of compounds were spotted on 6" × 17" sheets and chromatographed as for paper. Components on the guide strips were detected with alkaline permanganate. Elution of the purified oligosaccharides was carried out through Darco–Celite as described for paper. The phosphate extracted from the glass fiber sheet was not retained by the charcoal, and elution with water was continued until the Fiske and SubbaRow[22] test for phosphate[23] was negative. The oligosaccharides then were eluted from the carbon with 25% ethanol.

Reducing sugar,[19] hexosamine,[14,24,25] N-acetylhexosamine,[26] methylpentose,[27] galactose,[28] ethanol[29] and formaldehyde[30] determinations were performed as previously described[14,23] except that the galactose and hexosamine volumes were reduced to one-fifth and one-half, respectively.

Reduction of oligosaccharides was carried out with sodium borohydride (10 to 25 mg./ml.) at 0 to 4° overnight. The solution was then tested[31] to ascertain that an excess of borohydride was present. For most analytical procedures, acidification with HCl and evaporation to dryness three times with small volumes of methanol is sufficient to remove all but minute traces of boric acid. For periodate studies, however, the excess sodium borohydride is decomposed with Dowex-50(H^+) which also removes sodium ions.

Periodate oxidation of the purified oligosaccharides was performed in 0.25 to 0.5 ml. volume containing 8.0×10^{-3} *M* sodium metaperiodate ($NaIO_4$) as previously described.[16] The solutions were kept at 0 to 4°. Periodate was measured by the method of Fleury and Lange,[32] except that the volumes were greatly reduced. A sample, 20 μl., is removed from the periodate reaction mixture and an equal volume of a solution containing *M*/100 sodium arsenite, *M*/10 potassium iodide and *M*/10 sodium bicarbonate is added. After 15 minutes at room temperature, the excess arsenite is titrated with *M*/1000 iodine using one drop of starch indicator.[33] A convenient inexpensive microburet can be constructed by drawing out the tip of a 0.2 ml. serological pipette and attaching a Clay Adams Pipette Suction Apparatus (A-2473) to the top. The entire assembly is mounted vertically and the buret calibrated with mercury. The buret can be cleaned with dichromate–sulfuric acid solution before use. Titration of 20 μl. *M*/100 arsenite requires 200 μl. *M*/1000 iodine. It has been found that titrations of this size have a precision of about 1% (±1 μl). Formic acid can be measured after conversion of the excess periodate to iodate with ethylene glycol, addition of excess iodide and a known excess of arsenite. The arsenite is then back titrated with *M*/1000 iodine. In order to ensure a sufficient volume for titration, an aliquot of about 100 μl. is required for measuring formic acid. Controls included periodate with no sugar and periodate with erythritol in each experiment.

Immunochemical Studies.—Inhibition of precipitation by oligosaccharides was measured as previously described[3] using the micro quantitative precipitin method[34] and developing color by the Folin–Ciocalteu tyrosine reagent.[33,34] One half ml. of human anti-A 59–113[35] was incubated at 37° for 0.5 hr. with saline or inhibitor (0.5 ml.), after which one half ml. hog 30 (A) (40 μg./ml.) was added. After 1 hr. at 37° the tubes were placed in the refrigerator for one week with mixing twice daily, centrifuged, washed and analyzed.[23] In one case, one third of these volumes of antigen, antiserum and inhibitor were used, and the washed precipitates were dissolved in alkali, made up to 2.0 ml. using one fifth the quantities of reagents.

Results

Figure 1 shows the chromatography on Darco–Celite of 2.46 g. of dialysate consisting of 1.05 g.

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TABLE I

PER CENT. COMPOSITION OF ISOLATED FRACTIONS[a]

Fraction	Amt. isolated, mg.	Reducing sugar[b] u	r	Galactose u	r	Hexosamine u	r	N-Acetylhexosamine	N-Acetylhexosamine after complete hydrolysis[c] and N-acetylation u	r[d]	$[\alpha]D^e$	Probable structure
A_2I_a	4.1	50	0	36	7	35	26	1	19	14	+120°	α-N-acetylgalactosaminoyl-(1 → 3)-galactose
	1.9	35	2	20	0	22	21	1				
A_2I_b	11.7	46	2	39	30	37	3	12	45	3	+ 23°	β-Galactosyl-(1 → 4)-N-acetylglucosamine
A_2II	39.8	58	0	42	32	42	2	44	45	2	+ 33°	β-Galactosyl-(1 → 3)-N-acetylglucosamine
A_5II	18.2	47	1	24	24	50	16	23	39	12	+110°[f]	α-N-acetylgalactosaminoyl-(1 → 3)-β-galactosyl-(1 → 3)-N-acetylglucosamine

[a] u and r represent untreated and borohydride reduced compounds. [b] Unhydrolyzed; glucose standard. [c] Hydrolyzed (2 *N* HCl 2 hr. 100°), evaporated to dryness and N-acetylated. [d] Reduction before hydrolysis. [e] Measured at room temperature at concentrations of 0.6 to 6.1 mg./ml. in a Keston Polarimetric Unit. [f] Values of 100°, 107° and 122° were obtained in three preparations.

*p*H 1.6 dialysate and 1.41 g. *p*H 1.75 dialysate. Preliminary column chromatography in this system of the *p*H 1.9, 1.75 and the 1.6 dialysates revealed an almost identical pattern and gross composition of the fractions with respect to reducing sugar, galactose, hexosamine, N-acetylhexosamine and inhibiting potency. The fractions emerging in the oligosaccharide region of the chromatogram, following the N-acetylhexosamine peak, were designated A_1 to A_{11} in order of elution. A_2 contained the most material, 220 mg.; A_5 was the most active fraction, a 2 mg. sample giving 68% inhibition of A-anti-A precipitation. On Whatman paper all fractions showed several components. A_2 and A_5 each could be separated into two fractions, the faster component being designated A_2II and A_5II and the slower A_2I and A_5I, respectively. About half the weight of A_2 and A_5 were found in each of their subfractions. By chromatography on glass fiber sheets and by electrophoresis on glass fiber, A_2II and A_5II behaved as single compounds; A_2I and A_5I were mixtures. A_2I could be separated into two components by preparative chromatography on glass fiber sheets; the faster component was designated A_2I_b and the slower A_2I_a.

The analytical properties of the isolated compounds are given in Table I. The values recorded are averages of 2 to 7 single determinations performed on sequential small preparations from the main peaks, except for one, A_2I_a, of which only 1.9 mg. was isolated.

The potency of the fractions in inhibiting A anti-A precipitation is shown in Fig. 2. It can be seen that of the three compounds isolated from peak A_2 only A_2I_a was active. From the data in Table I this active compound contains equal amounts of galactose and hexosamine. On reduction with sodium borohydride the galactose is reduced by 81% whereas the hexosamine is affected to a much lesser extent, 26%. In an earlier preparation from which only 1.9 mg. of A_2I_a was isolated all the galactose was reduced and the hexosamine was unaffected. Neither of the preparations of A_2I_a gives a N-acetylhexosamine reaction. After hydrolysis and N-acetylation a value for N-acetylhexosamine is obtained indicative of N-acetylgalactosamine. This was confirmed by ninhydrin degradation which yielded only lyxose with no arabinose detectable by chromatography.[36,37]

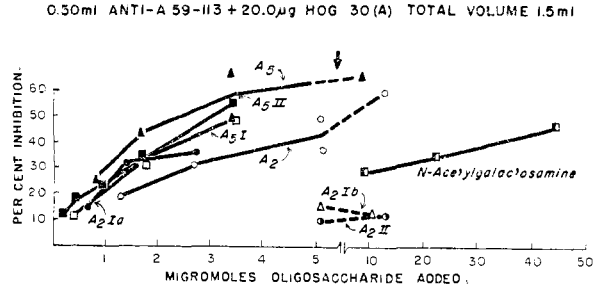
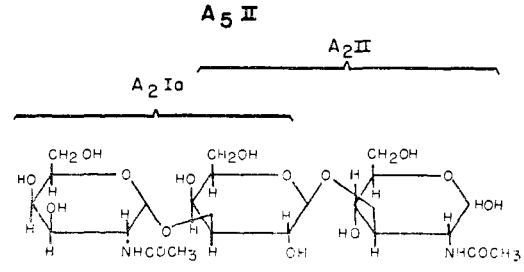


Fig. 2.—Inhibition by various fractions of A anti-A precipitation.

Thus A_2I_a is largely N-acetylgalactosaminoyl-galactose. The non-reducible galactose and reducible hexosamine could in part be explained by contamination with A_2I_b and in part by analytical variation on the small samples used. However,



since the two preparations of A_2I_a both had about the same potency in inhibiting A-anti-A precipitation and since one had no reducible hexosamine and no non-reducible galactose, it is clear that even if there is some contamination, the contaminant is not related to activity.

As can be seen in Table II, formaldehyde released by periodate oxidation before and after re-

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TABLE II

PERIODATE OXIDATION OF COMPOUNDS ISOLATED

Fraction	Condition	Periodate consumed[a] 5 days	Periodate consumed[a] 13 days	Periodate consumed[a] Theory	Formaldehyde released[a] 5 days	Formaldehyde released[a] 13 days	Formaldehyde released[a] Theory
A_2I_a	Unreduced	3.3	3.9	4.0	0.6	0.8	1.0
A_2I_b	Unreduced	3.0	3.4	3.0	.5	.7	1.0
A_2II	Unreduced	3.0	3.3	4.0	.6	.9	1.0
A_5II	Unreduced	2.7	2.8	3.0	.9	1.2	1.0
Fraction	Condition	4 hr.	6.5 hr.	Theory	4 hr.	6.5 hr.	Theory
A_2I_a	Reduced	3.4	3.5	4.0	1.7	1.8	2.0
A_2I_b	Reduced	3.1	3.1	3.0	1.2	1.1	1.0
A_2II	Reduced	2.7	2.6	4.0	0.9	0.9	1.0
A_5II	Reduced	2.5	2.4	3.0	1.1	1.1	1.0
β-Galactosyl-(1 → 3)-N-acetylglucosamine[c]	Reduced	2.6	2.5	4.0	0.9	0.9	1.0
Erythritol	Reduced	2.5	2.5	3.0	2.0[b]	2.0[b]	

[a] Reported as moles periodate consumed or moles formaldehyde produced per mole compound. [b] Formaldehyde released from erythritol used as standard. [c] Kindly supplied by Dr. F. Zilliken.

duction with $NaBH_4$ amounted to 0.8 and 1.8 moles, respectively, per mole of A_2I_a. Thus the N-acetylgalactosamine cannot be linked to carbons 1, 2, 5 or 6 of galactose, leaving linkage to carbons 3 and 4 as the only possibilities. To differentiate between these alternatives, it was reasoned that if the galactose on the reducing end of the molecule were substituted on carbon 3, it would be rapidly oxidized with periodate between carbons 1 and 2 to produce a formyl ester on carbon 5. The resulting lyxose thus would be protected against further periodate oxidation by the formyl ester on carbon 5 and the N-acetylgalactosamine residue on carbon 3. The lyxose formed then could be determined as pentose.[38] Periodate oxidation of a carbon 4-substituted analog would give a tetrose since oxidation between carbons 1, 2 and 3 would proceed readily. A_2I_a, 47 μg., was oxidized with 0.4 μmole of IO_4 in a volume of 60 μl. at room temperature for 0.5 hr. As a control, a compound with oxidizable galactose which should not give lyxose was treated similarly. A_2I_b, 50 μg., was chosen since it was isolated from the same fraction as A_2I_a and, as can be seen from Table I, probably is galactosyl-(1 → 4)-β-N-acetylglucosamine. The reaction was stopped byaddition of 40 μmole of arsenite in 10 μl. Water, 150 μl. was added after 10 minutes followed by 0.8 ml. of concentrated H_2SO_4 (without cooling), then 20 μl. of 3% cysteine. The reaction mixture was read at 3900 and 4240 Å. after 20 minutes standing at room temperature. Lyxose was used as a standard. Samples of A_2I_b and A_2I_a to which a periodate solution pretreated with arsenite was added served as additional controls. Only the periodate oxidized A_2I_a gave a positive reaction for pentose, 55% of one mole per mole of oxidized disaccharide. Non-oxidized A_2I_a, A_2I_b and oxidized A_2I_b gave no significant absorption for pentose. In view of the instability of formyl esters, a yield of lyxose of this order would be expected for a 3-linked reducing galactose. Thus compound A_2I_a is N-acetylgalactosaminoyl-(1 → 3)-galactose. The specific rotation of +120° indicates an α linkage. Since only 80% of the weight can be accounted for by the sum of the sugar components, the corrected specific rotation would be +150°.

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A_2I_b contains equal amounts of galactose and hexosamine. The hexosamine is on the reducing end of the molecule. The hexosamine is glucosamine from the N-acetylhexosamine obtained after hydrolysis and N-acetylation and by ninhydrin degradation to arabinose and chromatography. The low value, 12%, for N-acetylhexosamine of A_2I_b indicates[39] that the compound is primarily galactosyl-(1 → 4)-N-acetyglucosamine with perhaps some contamination with A_2II. The specific rotation of +23° is indicative of a β linkage. β-Galactosyl-(1→4)-N-acetylglucosamine has been isolated many times previously.[6,40-42]

A_2II also has equal amounts of galactose and hexosamine with glucosamine on the reducing end. Since A_2II gives the expected N-acetylhexosamine reaction, it must have an unsubstituted hydroxyl[39] on C-4. Periodate oxidation before and after reduction with sodium borohydride liberates 0.9 mole of formaldehyde, one mole of which would be expected for galactosyl-(1 → 3)-N-acetylglucosamine. The only other possible structure, galactosyl-(1 → 6)-N-acetylglucosamine, does not liberate any formaldehyde. The specific rotation of +33° indicates a β linkage. β-Galactosyl-(1 → 3)-N-acetylglucosamine has been isolated previously,[6,43] and a sample obtained by enzymatic synthesis[44] gave identical periodate uptake to the A_2II, although less than theoretical at 0° (Table II).

A_5II is composed of 2 moles of hexosamine and one of galactose. After complete hydrolysis, reaction with ninhydrin as well as N-acetylation and analysis for N-acetylhexosamine show the presence of both glucosamine and galactosamine. After borohydride reduction, hydrolysis and N-acetylation a value for N-acetylhexosamine is obtained indicating that, in the original trisaccharide, N-acetylglucosamine is on the reducing end of the molecule and N-acetylgalactosamine is non-reduc-

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(43) R. Kuhn, H. H. Baer and A. Gauhe, *Chem. Ber.*, **87**, 1553 (1954).

(44) Kindly supplied by Dr. F. Zilliken.

ible. A_5II also gives an N-acetylhexosamine color consistent with N-acetylglucosamine on the reducing end and indicating that the hydroxyl on C-4 is unsubstituted.[39] From Table II it can be seen that before and after reduction with sodium borohydride, A_5II yields one mole of formaldehyde (1.2 and 1.1, respectively) on oxidation with periodate. Thus C-6 of the N-acetylglucosamine on the reducing end of the trisaccharide must bear an unsubstituted hydroxyl. Therefore, only the hydroxyl on C-3 is available for substitution and hence A_5II must be a linear trisaccharide. Since A_5II is active in inhibiting A-anti-A precipitation, as is the disaccharide A_2I_a, α-N-acetylgalactosaminoyl-(1 → 3)-galactose, it was thought likely that of the fourteen possible arrangements of linking together galactose and N-acetylgalactosamine the one found in A_2I_a would be the most likely. Periodate studies support this contention. A_5II consumes only 3 moles of periodate (2.8 moles before reduction and 2.5 moles after reduction). Since 2 moles of periodate are required for the oxidation of the reducing unit, only one mole of periodate is consumed by the non-reducing and centrally located sugar units. The only structure which can satisfy this condition is N-acetylgalactosaminoyl-(1 → 3)-galactosyl-(1 → 3)-N-acetylglucosamine. Support for this conclusion is found in that less than one mole of formic acid is released on periodate oxidation and 65 to 70% of the galactose in the trisaccharide still can be measured after 6 days of exposure to an excess of periodate. The 30 to 35% loss is attributed to loss in manipulations involved in deionization through various resins (Amberlite MB-3 or IR-45 and Dowex 50) after periodate oxidation prior to the galactose determination. Thus it appears that A_5II has a nonreducing end similar to the active disaccharide, the α-N-acetylgalactosaminoyl-(1 → 3)-galactose. Since the specific rotation of the trisaccharide is less than that of the active disaccharide, the second linkage probably is β. Thus A_5II would be α-N-acetylgalactosaminoyl-(1→3)-β-galactosyl-(1→3)-N-acetylglucosamine.

Discussion

The trisaccharide isolated by mild acid hydrolysis of blood group A substance from human pseudomucinous ovarian cyst fluid has been inferred to have the structure α-N-acetylgalactosaminoyl-(1→3)-β-galactosyl-(1→3)-N-acetylglucosamine. Activity assays show that it is more active than the most active disaccharide previously reported,[6] α-N-acetylgalactosaminoyl-(1→3)-galactose, the compound representing the first two units from the non-reducing end (A_2I_a in this study). The disaccharide representing the two sugars at the reducing end was also isolated in both laboratories. It may be concluded, therefore, that this represents the sequence of the first three sugars from the non-reducing end of the oligosaccharide which is the antigenic determinant of blood group A specificity. Another fraction A_5I of activity comparable to the active trisaccharide A_5II was also isolated (Fig. 2). This material has not yet been obtained in pure form. However, its very low mobility on paper chromatography suggests that it may be larger than a trisaccharide, perhaps a tetrasaccharide. Since A_5I appears to consist of equimolar amounts of galactose and hexosamine, it may represent the first four units of the blood group A antigenic determinant. Such a tetrasaccharide could thus consist of the trisaccharide isolated above, A_5II, with an additional galactose on the reducing end. Such an alternating sequence of hexosamine and galactose is rendered more probable since none of the oligosaccharides isolated by Côté and Morgan or in this study have contained a hexosamine–hexosamine or a galactose–galactose sequence. Further studies on the characterization of A_5I are in progress.

The findings to date have given no insight into the basis for the subgroups of A since all of the active oligosaccharides fit into a single antigenic determinant. The smaller increment in inhibiting potency noted in comparing the active trisaccharide A_5II with the active disaccharide A_2I_a to that of the disaccharide A_2I_a relative to N-acetylgalactosamine is in accord with findings in many antigen-antibody systems that the terminal sugar or amino acid residue makes the highest contribution to the total free energy of binding and that the increment decreases continuously with increasing chain length.[23,45]

Addendum.—Professor W. T. J. Morgan reported in a lecture at the Rockefeller Institute in May, 1961, that the same trisaccharide as well as another trisaccharide in which the linkage of the galactose to the N-acetylglucosamine was β-1→4 had been isolated in his laboratory.[46]

(45) E. A. Kabat, *J. Immunol.*, **77**, 377 (1956); *J. Cellular Comp. Physiol. Supplement*, **50**, 79 (1957).

(46) I. A. F. L. Cheese and W. T. J. Morgan, *Nature*, **191**, 149 (1961).