recrystallized from the same solvent; yield of palmitamide, 24 mg; mp, 103–105°.

Periodate Oxidation of 16-Hydroxy-17-ketodotriacontane.—Compound I (200 mg) was oxidized in the same manner as described for compound V; yield of palmitylthiosemicarbazone, 96 mg; mp, 106–107°; yield of palmitic acid, 27 mg; mp, 62°.

Palmitaldehyde-1,2-^3H via ^3H_2O.—Either compound I or II (200 mg) was treated as described in the synthesis of compound III except that 10 mc (0.1 ml) of ^3H_2O was added to the reaction mixture. The isolated glycol was degraded as described for compound V and the distribution of isotope in positions 1 and 2 of the aldehyde (Table I) was determined via the thiosemicarbazone and amide derivatives. The radioactive glycol was stored until needed at which time it was cleaved to the aldehyde with periodate.

Palmitaldehyde-1,2-^3H via [^3H]$LiAlH_4$.—Compound II (252 mg) and 5.0 mg of [^3H]$LiAlH_4$ (25 mc) in 50 ml of dry ether were refluxed for 12 hours; unlabeled $LiAlH_4$ was added until the yellow color of the diketone was discharged. The reaction mixture was refluxed for 6 hours and the remainder of the procedure was carried out as described for compound IV; yield, 206 mg; mp, 97–107°.

The glycol was degraded as described for compound V with the exception that the *n*-heptane extract containing the palmitaldehyde was washed with several 5-ml portions of 0.1 N NaOH before the final wash with water. The distribution of activity (Table I) was determined as described for compound V.

14-Hydroxy-15-ketooctacosane (VI).—This compound was prepared from 7.6 g of Na and 40 g of methyl myristate in the same manner as described for the preparation of compound I; yield, 14.7 g; mp, 70–71°.

Anal. Calcd. for $C_{28}H_{56}O_2$ (424.4): C, 79.16; H, 13.30. Found: C, 79.06; H, 13.20.

The 2,4-dinitrophenylosazone melted at 122–123°.

Anal. Calcd. for $C_{40}H_{62}O_8N_8$ (782.5): N, 14.31. Found: N, 14.13.

14,15-Diketooctacosane.—This compound was prepared from 1 g of compound VI according to the procedure described for the preparation of compound II; yield, 860 mg; mp, 81–82°.

Anal. Calcd. for $C_{28}H_{54}O_2$ (422.4): C, 79.54; H, 12.89; Active H, 0. Found: C, 80.02; H, 13.06; Active H, 0.

12-Hydroxy-13-ketotetracosane (VII).—This compound was prepared from 8.6 g of Na and 40 g of methyl laurate under the conditions described for the preparation of compound I; yield, 19.2 g; mp, 61–62°.

Anal. Calcd. for $C_{24}H_{48}O_2$ (368.4): C, 78.18; H, 13.13. Found: C, 78.38; H, 12.99.

The 2,4-dinitrophenylosazone melted at 163–164°.

Anal. Calcd. for $C_{36}H_{54}O_8N_8$ (726.4): N, 15.42. Found: N, 15.12.

12,13-Diketotetracosane.—This compound was prepared from 1 g of compound VII according to the procedure described for the preparation of compound II; yield, 680 mg; mp, 72–73°.

Anal. Calcd. for $C_{24}H_{46}O_2$ (366.4): C, 78.61; H, 12.66; Active H, 0. Found: C, 79.01; H, 12.67; Active H, 0.

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Immunochemical Studies on Blood Groups. XXXII. Immunochemical Properties of and Possible Partial Structures for the Blood Group A, B, and H Antigenic Determinants*

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The immunochemical properties of oligosaccharide fragments A_3, B_3, and H_4, obtained from human ovarian cyst blood group A, B, and H substances by cleavage with NaOH in the presence of $NaBH_4$, have been studied. These fragments account with one exception for all the immunochemical reactivities of the intact blood group substances, including P1 specificity previously thought to reside in the interior of the molecule. Possible partial structures for the A, B, and H determinants are inferred.

In an earlier study (Schiffman *et al.*, 1964), a method for cleaving blood group mucopolysaccharides using NaOH in the presence of sodium borohydride was described. Paper chromatography of the fragments obtained yielded partially purified compounds having the properties expected for the antigenic determinants of blood group A, B, and H substances. These prod-

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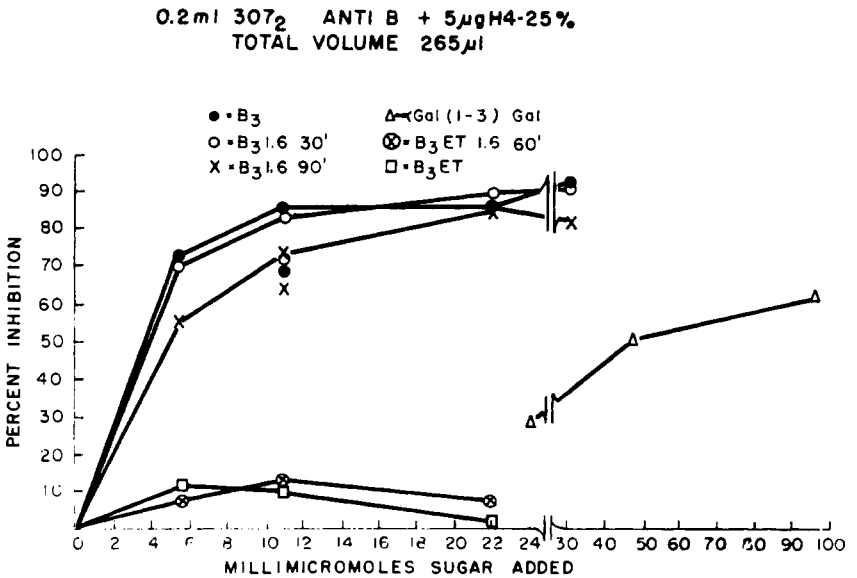

FIG. 1.—Inhibition of B-anti-B precipitation.

ucts, A_3, B_3, and H_4, all contained galactose, fucose and *N*-acetylglucosamine; A_3 contained *N*-acetylgalactosamine in addition; all contained small amounts of nitrogenous and possibly of other impurities. Based on the relative amounts of each sugar, A_3 and B_3 were judged to be about hexasaccharides. The largest units isolated thus far from blood group A substances are the two trisaccharides: α-*N*-acetylgalactosaminoyl-(1–3)-β-galactosyl-(1–3)-*N*-acetylglucosamine (A_3II) (Schiffman and Kabat, 1961; Cheese and Morgan, 1961; Schiffman *et al.*, 1962) and α-*N*-acetylgalactosaminoyl - (1–3) - β - galactosyl - (1–4) - *N* - acetylglucosamine (Cheese and Morgan, 1961). More recently a fucose containing oligosaccharide was reported by Morgan (1962). From blood group B substance the largest units previously reported (Painter *et al.*, 1963b) were also two trisaccharides: α-galactosyl-(1–3)-β-galactosyl-(1–3)-*N*-acetylglucosamine and α-galactosyl-(1–3)-β-galactosyl-(1–4)-acetylglucosamine. No compounds with H activity have been isolated and the structures determining H specificity, except for the involvement of a fucosyl residue, are largely unknown (Morgan, 1960a,b; Springer *et al.*, 1956; Springer and Williamson, 1962, 1963; Kabat, 1962b).

Watkins (1958, 1959), Ceppellini (1959) and Watkins and Morgan (1959) have formulated a scheme to explain the genetic control of the biosynthesis of blood group mucopolysaccharides. They postulate a common mucopolysaccharide precursor possessing the capacity to cross react with type XIV antipneumococcal antibody. This mucopolysaccharide is then assumed by a series of enzymic biosyntheses each of which is under genetic control to give rise sequentially to substances with Le^a activity, with H activity, and with A or B activity. The reverse sequence of reactions has been shown to occur by a series of enzymatic degradations. Thus, Iseki and Masaki (1953) and Iseki and Ikeda (1956) showed that blood group A or B substances treated with bacterial enzymes acquired H specificity. Although Howe *et al.* (1958) could not demonstrate an increase in H activity following digestion of blood group A substance with enzyme preparations from the same strain of *Clostridium tertium*, this transformation was found by Watkins (1960) using enzyme from *Trichomonas foetus*. The development of H activity by B substance was also reported by Iseki *et al.* (1959) using extracts from *Clostridium maebashi*, by Watkins (1956, 1962) using extracts from *T. foetus*, and by Watkins *et al.* (1962) using an α-galactosidase produced from coffee beans. The conversion of H substance into Le^a-active material (Watkins, 1960, 1962) and also into a substance with pneumococcal type XIV activity (Watkins, 1956) has also been obtained using *T. foetus* enzymes.

Many of these enzymatic conversions have been shown to occur with the loss of a single sugar. For example, coffee bean α-galactosidase acts on B substance to destroy B activity, to produce H activity, and only galactose is released in appreciable amounts (Zarnitz and Kabat, 1960; Watkins *et al.*, 1962). It seems reasonable, therefore, to postulate that the genetically controlled biosynthesis as outlined above may proceed by sequential addition of single sugars. As each sugar is added to the structure, the original activity may be largely masked and a new activity may appear.

Similar mechanisms may be operative in group A and C streptococcal carbohydrates and their variants (McCarty, 1960; Krause and McCarty, 1961). In studies of lysogenic conversion in group E *Salmonella*, Robbins and Uchida (1962a,b; Uchida *et al.*, 1963) have postulated that addition of glucose to a galactosyl-mannosyl-rhamnose structure gives rise to a new determinant, 34, in the lipopolysaccharide O antigen.

The present study offers support for this interpretation based on the immunochemical behavior of the purified fractions A_3, B_3, and H_4 derived from blood group A, B, and H substances, respectively. In addition an important body of experimental data on the P1 fractions (cf. Kabat, 1962a), the nondialyzable fractions obtained by mild acid hydrolysis of blood group A, B, and H substances, which did not seem to fit with the proposed biosynthetic scheme (Ceppellini, 1959; Watkins, 1958, 1959; Watkins and Morgan, 1959) has now been shown to be fully in accord with it. These P1 fractions showed little or no blood group activity and an increased cross reaction with type XIV antipneumococcal serum. Since the dialyzable fragments obtained on mild acid hydrolysis (Kabat and Leskowitz, 1955; Schiffman *et al.*, 1962) showed blood group activity, the P1 fractions were considered to represent groupings in the interior of the molecule. The present study shows that A_3 and B_3, which possess blood group activity, develop the capacity when exposed to mild acid hydrolysis to inhibit

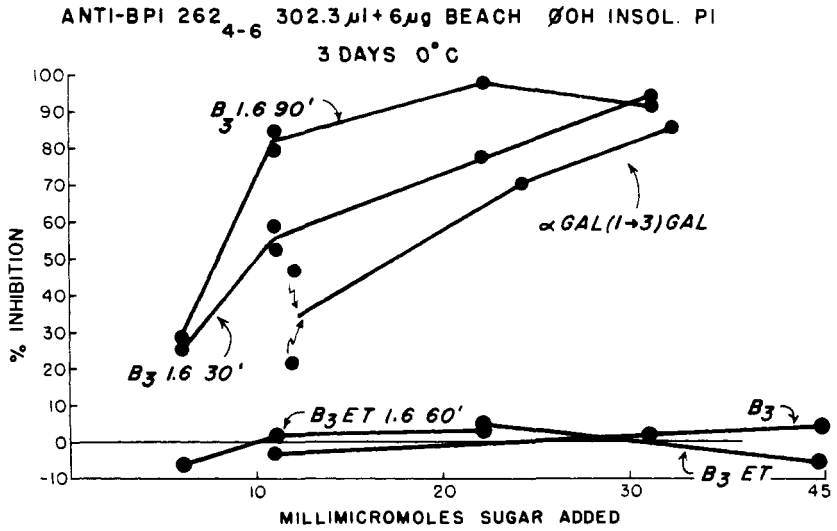

FIG. 2.—Inhibition of BP1-anti-BP1 precipitation.

the AP1-anti-AP1 and the BP1-anti-BP1 reactions, respectively, indicating that P1 specificity is contained within the A and B determinants themselves, and that removal of fucose from the determinants is responsible for the appearance of P1 specificity. From the groupings known to be involved in these various activities, certain postulations as to the structure of the A, B, and H antigenic determinants are advanced.

MATERIALS AND METHODS

Materials and methods were as described in a previous paper (Schiffman *et al.*, 1964). A_3, B_3, and H_4 are the products previously isolated from human ovarian cyst fluids MSS (A), Beach (B), and JS(H) (Schiffman *et al.*, 1964).

EXPERIMENTAL AND RESULTS

B_3 purified by paper chromatography is composed of approximately equimolar amounts of fucose, galactose, and *N*-acetylglucosamine. Using extracts of *Ulex europeus* seeds, blood group O cells can be agglutinated. This agglutination is inhibited by blood group H active compounds.

B_3 (2.0 ml) was incubated with 0.2 ml coffee bean extract and 0.2 ml 0.1 M acetate buffer, *p*H 4.8, at 37° for 18 hours and tested for its ability to inhibit *Ulex* agglutination of O cells. The results are given in Table I.

B_3 was hydrolyzed at 95° at *p*H 1.6 for 30 and 90 minutes and α-galactosidase-treated B_3 (B_3ET) was hydrolyzed for 60 minutes under the same conditions. After neutralization the solutions were tested for their ability to inhibit precipitation of anti-B by blood group B substance, of anti-BP1 by BP1 substances, and of type XIV antipneumococcal antibody by BP1 substance. The results are presented in Figures 1, 2, and 3. Calculations of the millimicromoles of sugar added are based on the initial concentrations of oligosaccharide in B_3 and B_3ET so that the hydrolyzed samples B_3 1.6, 30 minutes, B_3 1.6, 90 minutes, and B_3ET 1.6, 60 minutes are directly comparable to the original.

TABLE I

MINIMUM CONCENTRATION OF SUBSTANCES GIVING INHIBITION OF THE HEMAGGLUTINATION OF O ERYTHROCYTES BY AN EXTRACT OF *Ulex europeus*

Substance	Concentration (µg/ml)
H substance (JS)	4
B_3 untreated	2500
B_3 α-galactosidase-treated	63
α-galactosidase alone	No inhibition

In the B-anti-B system, Figure 1, treatment of B_3 with α-galactosidase almost completely destroyed its ability to inhibit and subsequent mild acid hydrolysis did not increase the inhibiting activity. The B activity of B_3, however, is only slightly diminished by mild acid hydrolysis; after 90 minutes only about 4.5 mµmoles are required for 50% inhibition as compared with the original value of about 3 mµmoles; it was still ten times as active as α-galactosyl-(1–3)-galactose.

The potency of these materials in inhibiting BP1-anti-BP1 precipitation is given in Figure 2. B_3 is completely inactive in inhibiting precipitation of anti-BP1 by a human BP1 substance. Exposure of B_3 to mild acid hydrolysis for only 30 minutes produces a material which is more active than α-galactosyl-(1–3)-galactose, the most potent inhibitor thus far reported (Kabat and Schiffman, 1962); hydrolysis for 90 minutes is even more effective. In sharp contrast, however, no P1-inhibiting activity developed on heating α-galactosidase treated B_3 for 60 minutes at *p*H 1.6 at 95°.

The findings as assayed by inhibition of precipitation of type XIV horse antipneumococcal serum by BP1 substance (cf. Kabat, 1962c) are presented in Figure 3. Neither B_3 nor B_3ET inhibit significantly. After mild hydrolysis α-galactosidase-treated B_3 becomes one of the most potent inhibitors. B_3 hydrolyzed for 30 minutes shows activity comparable to that of lactose and after 90 minutes becomes even more active.

Paper chromatographic investigation of the products obtained by hydrolysis of B_4L, a fraction with B activity but with only one-half the hexosamine of B_3, at *p*H 1.6 for 90 minutes at 95°, showed 8 components. Elution from the paper and analysis demonstrated that no fucose-containing oligosaccharides were present. All fucose was present as free monosaccharide. Several of the compounds had both galactose and hexosamine and were active in inhibiting B-anti-B and BP1-anti-SXIV precipitation.

Treatment of blood group B substances with coffee bean α-galactosidase produces H activity with a greatly diminished ability to inhibit and precipitate anti-B

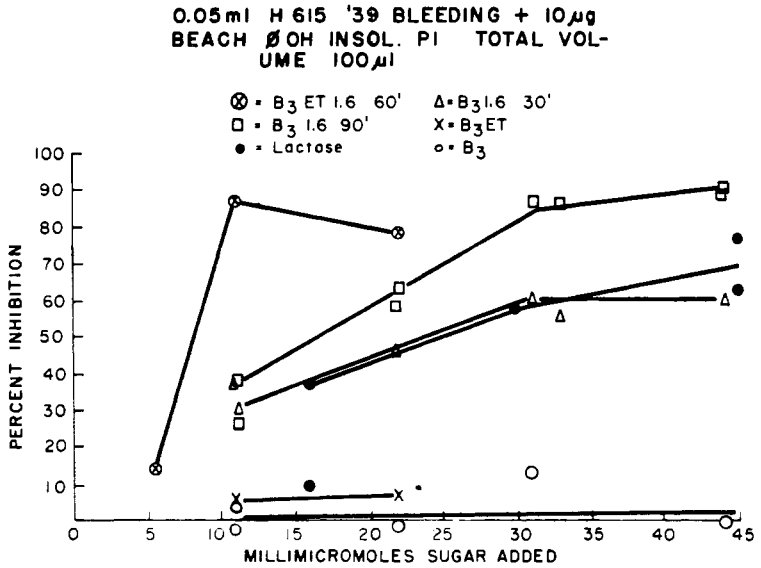

FIG. 3.—Inhibition of cross reactivity of BP1 substance with type XIV horse antipneumococcal serum.

(Zarnitz and Kabat, 1960; Watkins *et al.*, 1962). Treatment of B substance, Beach φOH-insoluble, with crude α-galactosidase resulted in a complete loss in ability to precipitate with one anti-B serum, 307_2, and a greatly diminished loss in ability to precipitate with another antiserum, 310_4. Although 2.6 μg untreated Beach φOH-insoluble precipitated 2.9 μg N from 101 μl antiserum 310_4, 2.6 μg α-galactosidase-treated Beach φOH-insoluble precipitated only 0.50 μg N, and 50 μg of the enzyme-treated substance was necessary to precipitate 1.97 μg N.

When α-galactosidase-treated Beach φOH-insoluble was hydrolyzed for 2 hours at *p*H 1.6 at 95° no BP1 activity was detected (2.6–10 μg ET Beach P1 failed to precipitate any antibody N), H activity was lost, and a greatly increased ability to precipitate horse antipneumococcal type XIV serum developed.

A_3 is composed of 2 moles of fucose, 1 mole galactose, 1 or 2 moles *N*-acetylglucosamine, and 1 mole of *N*-acetylgalactosamine. On mild acid hydrolysis A_3 behaves in A-anti-A precipitation in a manner very similar to that found for B_3 in the B-anti-B system. The information available on the structures associated with the antigenic determinants of AP1, the non-dialyzable residue after mild acid hydrolysis of blood group A substance, is extremely limited (Allen and Kabat, 1959). In analogy to the findings with B_3, it was anticipated that A_3 would be inactive in inhibiting precipitation of anti-AP1, that mild acid hydrolysis of A_3 would produce potent inhibition of AP1-anti-AP1, and that the linear trisaccharide A_5II would also be a good inhibitor. This proved to be correct. As can be seen in Table II, 20–30 μmoles of monosaccharide had to be added for significant inhibition, while only 0.05–0.1 μmole of A_5II or mildly hydrolyzed A_3 was needed. Among the monosaccharides, *N*-acetylgalactosamine which had not been tested previously by Allen and Kabat (1959) appeared somewhat better than *N*-acetylglucosamine.

Forssman activity is associated only with blood group A, not with B or H (O). The two activities, however, do not run parallel, since many types of treatment result in an increase in Forssman activity relative to blood group A activity (Landsteiner and Harte, 1940; Aminoff *et al.*, 1948). In a previous study A_3 was shown to be about 20 times better than A_5II as an inhibitor of Forssman activity. Figure 4 shows the Forssman activity of a blood group A substance on mild acid hydrolysis. About 30 mμg of unhydrolyzed blood group A substance, McDon (A), gave 50% inhibition of lysis. After 2 hours of hydrolysis at *p*H 1.9 (100°), and even after 2 more hours at *p*H 1.75, only about 20 mμg is required for the same degree of inhibition of lysis; hydrolysis at 100° for 2 hours at *p*H 1.6, however, results in loss of activity, 70 mμg being required. On the other hand, Figure 5 shows that the capacity of McDon (A) to inhibit lysis of human A red cells by the same rabbit antiserum is progressively diminished on mild acid hydrolysis.

Hydrolysis of A_3 at *p*H 1.6 for 15, 30, and 60 minutes followed by neutralization of the solution, without isolation of the hydrolysis products, showed that 2 mμmoles of unhydrolyzed A_3 was sufficient to give 50% inhibition of sheep cell lysis as compared with 3, 4, and 6 mμmoles of A_3 after hydrolysis for 15, 30, and 60 minutes, respectively; 23 mμmoles of A_5II was needed for 50% inhibition. A_3 hydrolyzed at *p*H 1.6 for 15, 30, 60, and 90 minutes at 95° develops the

TABLE II

INHIBITION BY A_3, ITS HYDROLYSIS PRODUCTS, AND VARIOUS SUGARS OF AP1-anti-AP1 PRECIPITATION

	Sugar Added (μmoles)	Inhibition (%)	Anti-AP1 Used (serum 306_4) (μliters)
Fucose	6.2	−11	302
	30.5	14	298
Galactose	5.4	−7	302
	27.6	30	298
N-Acetylglucosamine	4.5	1	302
	23.0	42	298
N-Acetylgalactosamine	4.7	11	302
	18.9	44	298
A_5II	0.036	28	395
	0.036	70	298
	0.093	91	298
	0.183	100	298
	0.183	85	302
A_3	0.036	−7	395
	0.084	−6	302
	0.183	15	298
A_3, *p*H 1.6, 95°, 90 minutes	0.036	64	395

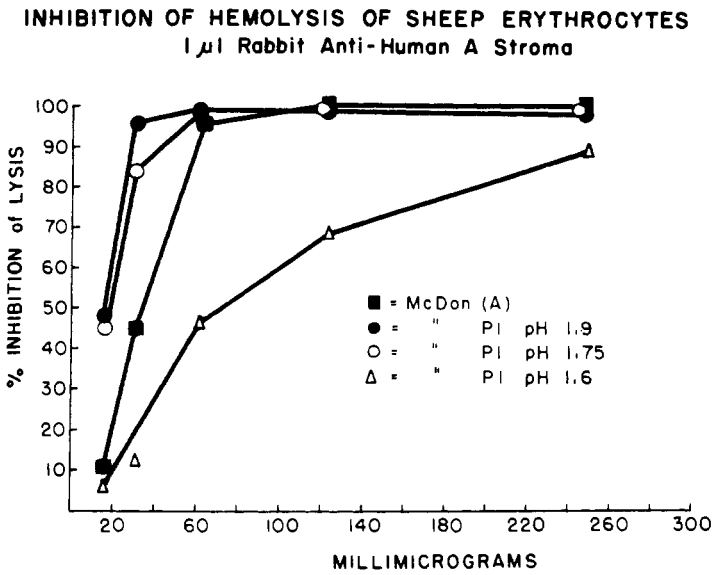

FIG. 4.—Effect of mild acid hydrolysis on Forssman activity of blood group A substance.

ability to inhibit the precipitation of type XIV horse antipneumococcal serum by BP1; unhydrolyzed A_3 did not do so significantly; 50 and 100 μg of A_3 inhibited precipitation 5 and 11%, respectively, while similar amounts of A_3 hydrolyzed at *p*H 1.6 for 90 minutes at 95° inhibited 42 and 54%, respectively, and 20 μg inhibited 91%.

H_4 is an oligosaccharide composed of *N*-acetylglucosamine and fucose in almost equal proportions but with somewhat less galactose. It inhibits hemagglutination of O cells by *Ulex* extracts some forty times better than does L-fucose on a weight basis. Hydrolysis at *p*H 1.6 for 90 minutes at 95° followed by paper-chromatographic separation of the fragments revealed that all the fucose was liberated as monosaccharide; galactose and *N*-acetylglucosamine in the hydrolysate occur mainly in oligosaccharide linkage.

Elution and analysis of the seven regions of the chromatogram darkened by silver revealed that most of the galactose and *N*-acetylglucosamine occurred in a 1:1 molar ratio in two regions with R_F values less than that of galactose, i.e., in the disaccharide area of the paper. These two components were susceptible to hydrolysis by a purified preparation of β-galactosidase (Marcus *et al.*, 1963). Unhydrolyzed H_4 is a poor inhibitor of the precipitation of anti-SXIV by degraded blood group substances. Ten to 20% inhibition was obtained when 25–100 μg H_4 was used. The two components obtained from H_4 after mild acid hydrolysis and paper chromatography, however, inhibited precipitation about as well as does β-galactosyl-(1–4)-*N*-acetylglucosamine, i.e., 20 μg of oligosaccharide is needed to give 70–80% inhibition.

DISCUSSION

Oligosaccharides A_3, B_3, and H_4 derived from human ovarian cyst blood group substances by alkaline cleavage in the presence of $NaBH_4$ are considered to be the determinants of antigenic specificity. Not only are these paper-chromatographically purified compounds more potent inhibitors than other oligosaccharides thus far reported but they possess, with one exception, every immunochemical reactivity of the intact blood group mucopolysaccharide for which they have been tested. Blood group B substance can be converted into an H active substance by α-galactosidase. This has been accomplished with B_3. Neither blood group A nor B substance will react with anti-AP1 or BP1. A_3 and B_3 do not inhibit anti-AP1 or anti-BP1. After mild acid hydrolysis blood group A and B substances will react with anti-AP1 and anti-BP1, respectively. After mild acid hydrolysis A_3 and B_3 become highly active in inhibiting precipitation of anti-AP1 and anti-BP1, respectively. α-Galactosidase-treated blood group B substance does not yield BP1 after mild acid hydrolysis nor does α-galactosidase-treated B_3 after mild acid hydrolysis inhibit BP1-anti-BP1 precipitation. Blood group A, B, and H substances react poorly with antipneumococcal type XIV serum, and A_3, B_3, and H_4 are poor inhibitors in this system. Blood group substances show a dramatic increase in ability to precipitate antipneumococcal type XIV serum after mild acid hydrolysis; A_3, B_3, and H_4 inhibit very well after mild acid hydrolysis. Forssman activity is associated with blood group A substance. A_3 is a potent inhibitor of Forssman activity.

What has not been attempted, for lack of enzymes, is the conversion of A_3 into an H-active oligosaccharide and H_4 into an Le^a-active compound.

Forssman activity of blood group A substance increases after hydrolysis at *p*H 1.9 and 1.75 for 2 hours at 100°. The ability of A_3 to inhibit lysis of sheep erythrocytes, however, decreases after hydrolysis at *p*H 1.6, 95°, for 15, 30, and 60 minutes. The reason for this is not clear.

Substantial evidence has been presented that intact A and B blood group substances differ in that the former contains a terminal *N*-acetylgalactosamine while the latter contains a terminal galactose (Kabat and Leskowitz, 1955; Watkins and Morgan, 1955; Gibbons *et al.*, 1955; Hiyama, 1962). Analytical data on A_3 and B_3 show this relationship to hold for the antigenic determinants. Moreover, galactose is split off with loss of activity from B_3, further indicating its terminal position; this results in the development of H activity. Accordingly the terminal α-galactosyl residue prevents the manifestation of H activity in B_3 to a large extent. With a sample of A substance from the serous fluid of an ovarian cyst (MSS), deacetylation of the terminal *N*-acetylgalactosamine by *Cl. tertium* enzyme did not result in H activity but subsequent removal of the terminal galactosamine by

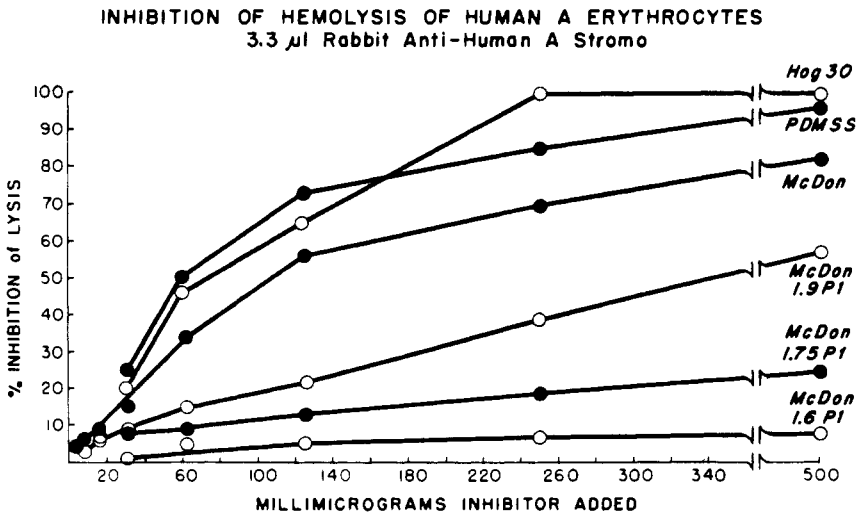

FIG. 5.- Effect of mild acid hydrolysis of blood group A substances on their capacity to inhibit hemolysis of A erythrocytes by rabbit anti-A.

deamination with nitrous acid yielded a product with high H activity. However, with blood group substance from the mucinous fluid of the same cyst (MSM), enzymic deacetylation resulted in a material with substantial H activity and this was not affected by reacetylation (Marcus *et al.*, 1964). Thus H activity appears to be blocked in A substance either by the entire terminal *N*-acetylgalactosamine residue or by its *N*-acetyl group.

An H determinant from the immunochemical data may thus be a molecule of A_2 or B_3 lacking a terminal *N*-acetylgalactosamine or a galactose, respectively. Analytical data on H_4 show it to have somewhat less galactose than B_3 and to have no *N*-acetylgalactosamine. If one calculates the analytical data (Schiffman *et al.*, 1964) on H_4 assuming 2 moles each of *N*-acetylglucosamine and methylpentose, the expected molar concentration of galactose should be 2.0–2.4 μM, the actual value found was 3.1 μM. It should be noted, however, that analytical data on another H-active fraction, H_3, and on three other B-active fractions give different molar compositions. Further purification and characterization of these materials is necessary.

Since A_3 and B_3 contain about 2 methylpentoses per molecule and on the assumption that one or another of the two active A and B trisaccharides previously isolated are represented in the determinants, the two fucose residues may be attached either to the central galactose, to the *N*-acetylglucosamine, or to both. An additional possibility, since A_3 and B_3 were obtained by alkaline borohydride, is that an additional sugar residue at the reducing end, which had been converted to a sugar alcohol, contains one or more of these residues. The best interpretation of the data available would be that one fucose which is involved in H specificity is linked to the subterminal galactose in A_3 and B_3. This would be consistent with the blocking of H activity by the terminal galactose in B_3 or *N*-acetylgalactosamine in A_3. It would also explain the appearance on mild acid hydrolysis of BP1 or AP1 activity. The stronger cross reactivity with type XIV antiserum by acid-hydrolyzed α-galactosidase-treated B_3 as compared with acid-hydrolyzed B_3 conforms well with this interpretation.

The second fucose may be hypothesized to be attached to the *N*-acetylglucosamine. This would be consistent with the interpretation of Watkins and Morgan (1957) from studies with the oligosaccharides of human milk (Kuhn, 1957) that a branched trisaccharide in which an α-fucosyl residue attached to carbon 4 of a β-galactosyl-1-3-*N*-acetylglucosamine is associated with Le^a specificity.

On this basis a possible partial structure of B_3 would be:

galactosyl α-1-3-galactosyl-β-1,3-N-acetylglucosaminoyl-
↑α or ↑α
fucosyl 1,4 fucosyl

A_3 by analogy would have a terminal *N*-acetylgalactosaminoyl residue linked α-1,3- to the middle galactose. An H determinant could be further hypothesized to be the structure minus the terminal galactose or *N*-acetylgalactosamine.

This formulation accounts very well for the data presented on the effects of mild acid hydrolysis on A_3, B_3, H_4, and α-galactosidase-treated B_3 in addition to the P1 findings presented above. The formulation also accounts for most of the oligosaccharides isolated thus far from the blood group substances and of all the active oligosaccharides from A and B substances (Côté and Morgan, 1956; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962; Cheese and Morgan, 1961; Painter *et al.*, 1962, 1963b).

Analytical data on A_3 and B_3 (Schiffman *et al.*, 1964) in their present impure state, indicate that they may contain an additional glucosamine residue. This could be attached either to the subterminal galactose or to a reduced sugar to which the *N*-acetylglucosaminoyl residue may be linked. The failure of Yosizawa (1961) to find two hexosamines linked to each other after hydrazinolysis suggests that the second *N*-acetylglucosamine would probably not be linked to another hexosamine, since hydrazinolysis deacetylates *N*-acetylhexosamines. Free hexosamines linked glycosidically to other residues are very resistant to acid hydrolysis (Stacey, 1958) so that hexosamine-hexosamine bonds if present would tend to accumulate.

If the additional *N*-acetylglucosamine of A_3 or B_3 were attached to the subterminal galactose in the structure given above, it could not account for the isolation by Yosizawa (1962) and by Painter *et al.* (1963a) of β-*N*-acetylglucosaminoyl-1,3-galactose in good yield from A, B, and H substances, since position 3 of this galactose is already occupied but could account for *N*-acetylglucosaminoyl-β-1-6-galactose (Yosizawa, 1962). However, if the reducing *N*-acetylglucosamine in the structure shown were linked on either

carbon 3 or carbon 6 to a galactose which had been reduced by borohydride, the additional *N*-acetylglucosamine might then be linked on carbon 6 or carbon 3, respectively. This would include in the structure the possibility of accounting for the isolation of both *N*-acetylglucosaminoyl-β-1,3 and β-1,6-galactoses but also of the branched trisaccharide:

N-acetylglucosaminoyl-β-1,3-galactose
$\uparrow \beta$-1,6
N-acetylglucosaminoyl

isolated from hydrazinolysates by Yosizawa (1962). The fraction A_5I mentioned by Schiffman *et al.* (1962) on further purification on a cellulose column using propanol-water (7:2) yielded a tetrasaccharide as active as A_5II with a galactose at the reducing end. The isolated product A_5Id contained 835 μg of galactose and 848 μg of hexosamine; after reduction with sodium borohydride the galactose fell to 461 μg while the hexosamine value was 715 μg.

It should be noted, since two active trisaccharides were isolated from A and from B substances, that the positions of the fucoses attached to the *N*-acetylglucosamine residues need not be the same. Indeed, even if they were attached in both instances to carbon 6 of the *N*-acetylglucosamine, their spatial orientation relative to the rest of the determinant would differ substantially. What effect such variations would have on the A, B, H, and Le^a activities remains to be determined when complete structures for each have been established.

Of especial import are the ability to reconcile these newer data on the P1 substances with proposed schemes of biosynthesis of the A, B, H, and Le^a substances (Watkins, 1958, 1959; Ceppellini, 1959; Watkins and Morgan, 1959). As long as the P1 groups were thought to be in the interior of the molecule and in view of the specificity differences found between AP1 and BP1, it appeared reasonable to attribute to the A and B genes biosynthetic functions apart from the addition of terminal α-*N*-acetylgalactosaminoyl and α-galactosyl residues to H-active material. The present findings account satisfactorily for the P1 data as being associated with the A and B determinants themselves in which AP1 and BP1 specificity are blocked by fucose residues and thus become fully consistent with proposed biosynthetic schemes.

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