Studies on Specificity and Binding Properties of the Blood Group A Reactive Hemagglutinin from *Helix pomatia**

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ABSTRACT: Equilibrium dialysis measurements on the interaction between purified *Helix pomatia* A hemagglutinin and a blood group A active reduced pentasaccharide (AR_L 0.52) showed the hemagglutinin to have six combining sites of intrinsic association constant of 5×10^3 l./mole at 25°. The binding data were linear in Scatchard and Sips plots and homogeneity of sites was also indicated from displacement experiments with Me-α-D-GalNAc and with weakly binding haptens (*e.g.*, Me-α-D-GNAc and Et-β-D-GNAc). In inhibiting precipitation of hemagglutinin by blood group A substance, the phenyl α-glycoside of D-GalNAc was as active and the nitrophenyl α-D-GalNAc was about one-half as active as D-GalNAc. Precipitation with group A and C streptococcal polysaccharides, and with native and desialized ovine submaxillary mucin, established that the hemagglutinin precipitates with macromolecules having terminal nonreducing α-linked D-GalNAc but not with those having β-linked D-GNAc end groups. Precipitation of hemagglutinin by blood group A substance was relatively insensitive to pH but precipitation by teichoic acid from *Staphylococcus aureus* strain 3528 was complete only within a narrow range around neutrality.

The concentration of D-GalNAc for 50% inhibition of precipitation was approximately 100 times greater for A substance than for teichoic acid and was correlated with the measured difference in binding of the hemagglutinin for the two types of reactive groups in the antigens (*e.g.*, α-linked D-GalNAc and α-linked D-GNAc end groups).

Studies from several laboratories have demonstrated that plants and invertebrates contain substances which specifically agglutinate red cells of human or animal origin (for reviews, see Mäkelä, 1957; Bird, 1959; and Prokop *et al.*, 1968). Prokop *et al.* (1965a,b) found that the albumin gland of the snail *Helix pomatia* contained a very potent blood group A specific agglutinin. This protein could readily be isolated in a highly purified state by absorption to insoluble blood group A + H substance and elution with D-GalNAc[1] (Hammarström and Kabat, 1969). The hemagglutinin was precipitated by macromolecules with terminal nonreducing α-linked D-GalNAc or α-linked D-GNAc residues (Hammarström and Kabat, 1969). As determined by inhibition of precipitation with low molecular weight carbohydrates, the combining site of the hemagglutinin was found to be only about as large as an α-linked 2-acetamido-2-deoxyhexopyranose residue. The methyl α-glycoside of *N*-acetyl-D-galactosamine was most potent but the site could accommodate the methyl α- or β-glycosides of *N*-acetyl-D-glucosamine although they were less strongly bound (Hammarström and Kabat, 1969).

The purpose of this investigation was threefold: (1) to define further the stereochemical requirements of the combining site, (2) to determine homogeneity or heterogeneity of sites, and (3) to evaluate the utility of the precipitin reaction between *Helix pomatia* A hemagglutinin and carbohydrate antigens in combination with inhibition studies for detecting particular end groups in macromolecules.

Materials

H. pomatia A hemagglutinin was prepared by absorption on insoluble hog blood group A + H substance and subsequent elution with D-GalNAc as described earlier (Hammarström and Kabat, 1969). The hemagglutinin was homogeneous as determined by ultracentrifugation and immunoelectrophoresis with antisera to the crude extract and was completely precipitated by human blood group A substance (Hammarström and Kabat, 1969). Streptococcal polysaccharides from groups A (strain B 401), A_{var} (strain T27 A_{var}), and C (strains K 64 and C 74) were gifts from Drs. R. M. Krause and M. McCarty (*cf.* Krause, 1963; Krause and McCarty, 1962). Teichoic acid from *Staphylococcus aureus* strain 3528 (Nathenson *et al.*, 1966) was a gift from Dr. J. L. Strominger. Poly(*N*-acetyl-D-glucosamine 1-phosphate) from *Staphylococcus lactis*, NCTC 2102 (Archibald *et al.*, 1968), was kindly provided by Dr. J. Baddiley, and *Salmonella typhimurium* strain LT-2 lipopolysaccharide (Hellerqvist *et al.*, 1969) by Dr. S. Svensson. Ovine submaxillary mucin (OSM) (Pigman and Gottschalk, 1966) was donated by Dr. W. Pigman; desialized ovine submaxillary mucin (DOSM) was prepared in the laboratory using neuraminidase from *Vibrio cholera* (Behringwerke AG, Germany). The *N*-acetylneuraminic acid content before and after neuraminidase treatment was 26.2 and 0.4%, respectively, as determined by the thiobarbituric acid method of Warren (1959). The

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[1] Abbreviations used are: D-GalNAc, *N*-acetyl-D-galactosamine; D-D-GNAc; *N*-acetyl-D-glucosamine; OSM, ovine submaxillary mucin; DOSM, desialized OSM.

blood group substances used were previously described from human ovarian cysts (*cf.* Kabat, 1956, and Schiffman *et al.*, 1964). Human blood group H substance (preparation JS), degraded by periodate oxidation, reduction, and hydrolysis (Goldstein *et al.*, 1965), has been described earlier (Lloyd and Kabat, 1968).

All carbohydrate antigens except for the *S. typhimurium* strain LT-2 lipopolysaccharide were analyzed for chemical composition by colorimetric methods. Analyses were in good agreement with those of the original authors.

Monosaccharides and their methyl or ethyl glycosides have been described earlier (Beychok and Kabat, 1965). Phenyl α-D-GalNAc, *o*- + *p*-NO_2Ph-α-D-GalNAc, and *p*-NO_2Ph-β-D-GalNAc were gifts from Dr. I. J. Goldstein. A reduced blood group A active pentasaccharide, AR_L 0.52, α-D-GalNAc-(1→3)-[α-L-Fuc-(1→2)]-β-D-Gal-(1→4)-β-D-GNAc-(1→6)-3-hexenetetrol(s), obtained by alkaline borohydride degradation of hog mucin blood group A + H substance, has been described earlier (Lloyd *et al.*, 1966; Moreno and Kabat, 1969). Tritium-labeled blood group A active reduced pentasaccharide, [^{3}H]AR_L 0.52 (specific activity 6.89 × 10^8 cpm/μmole), was that used by Moreno and Kabat (1969).

Methods

Analytical Methods. Colorimetric methods for nitrogen, hexosamine, *N*-acetylhexosamine, methylpentose, and hexose determinations have been described previously (Kabat, 1961; Lloyd *et al.*, 1966). Galactosamine was determined by the method of Ludowieg and Benmaman (1967), sialic acid by the thiobarbituric acid method (Warren, 1959), and phosphorus according to Fiske and Subbarow (1925) as modified by Bartlett (1959).

Immunological Methods. Hemagglutination was performed with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using 0.025-ml loops and 2% erythrocyte suspensions. Agglutination was recorded after 1-hr incubation at room temperature. Quantitative precipitin analyses (*cf.* Kabat, 1961) with about 4 μg of hemagglutinin N were carried out by a microprecipitin technique using the ninhydrin procedure for nitrogen determination (Schiffman *et al.*, 1964). Equilibrium dialysis and displacement experiments were carried out in glass cells (Kabat, 1961) manufactured by Büchler Instruments, Fort Lee, N. J. The cells were incubated at 25 ± 0.1°, for 72 hr; this time was sufficient to obtain equilibrium. The total volume per chamber was 1.00 ml and the buffer employed was 0.01 M phosphate (pH 7.3) containing 0.9% NaCl and 0.1% NaN_3. Aliquots from the side containing hapten only, in duplicate or triplicate, were taken and counted. The proportion of bound and free hapten in the cell was calculated from the total radioactive hapten added, the extent of binding to the membrane, and the concentration of free hapten in the protein free compartment. Binding to the membrane was approximately 7%, and was independent of the concentration of nonradioactive hapten within the range of the experiment. Detailed descriptions of equilibrium dialysis have been given (Kabat, 1961; Weir, 1967). Radioactivity measurements for some cells containing high concentrations of hapten were also checked by colorimetric determination of methylpentose (aliquots were treated with ion exchanger, Dowex 1-X8, OH^- form, to remove phosphate ions before analysis). Values for bound and free hapten were in good agreement with those obtained by radioactivity measurements.

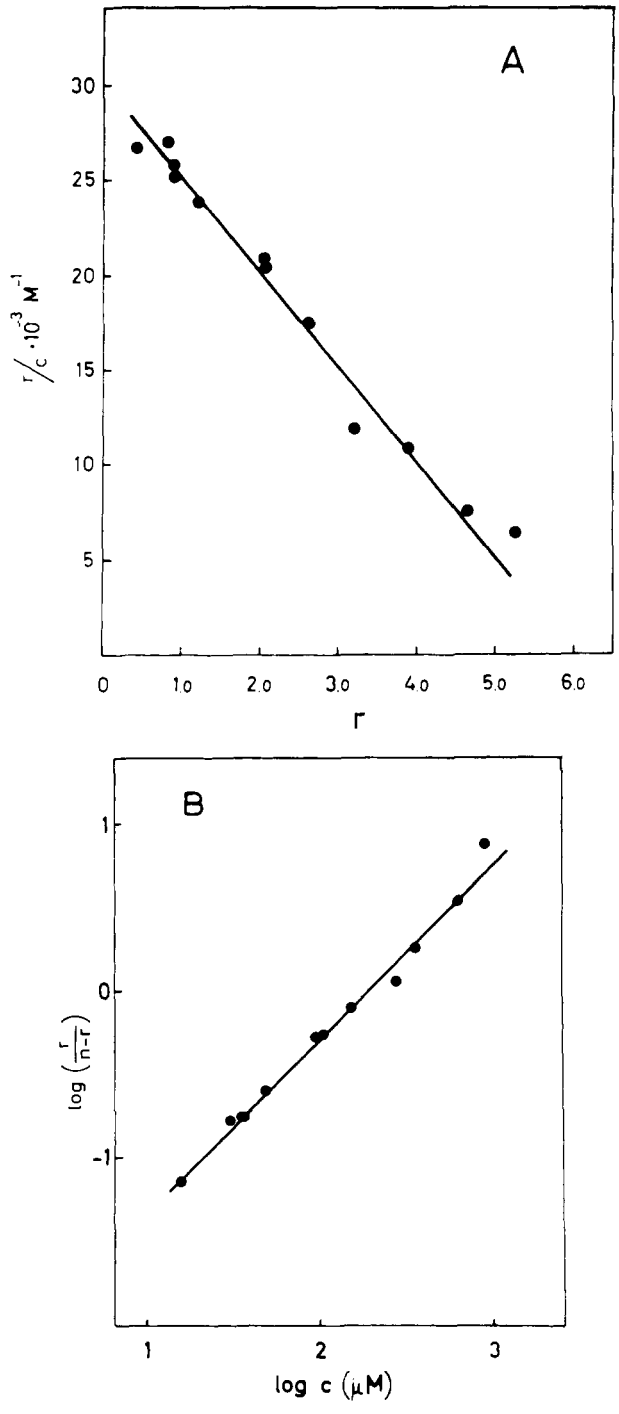

FIGURE 1: Equilibrium dialysis experiments with purified *Helix pomatia* A hemagglutinin and tritium labeled A active reduced pentasaccharide [^{3}H]AR_L 0.52. The data are plotted both according to Scatchard (A) and according to Sips (B). Approximately 12 mg of snail protein was used for each determination. For further details, see Materials.

Experimental Section and Results

Equilibrium Dialysis. Binding of ^{3}H-labeled A-active reduced pentasaccharide, AR_L 0.52, to purified *H. pomatia* A hemagglutinin at 25.0 ± 0.1° and pH 7.3 is shown in Figure 1. The binding data are plotted both according to Scatchard (1949) ($r/c = nK_0 - rK_0$, where r is moles of bound hapten per mole of hemagglutinin, c is free hapten concentration, K_0 the intrinsic association constant, and n the number

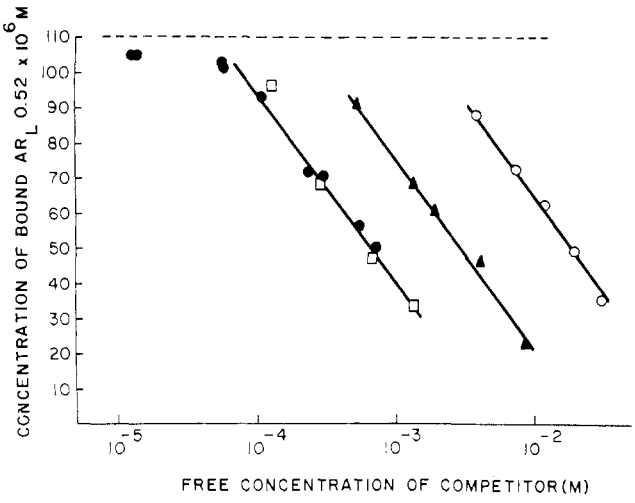

FIGURE 2: Equilibrium dialysis displacement experiments with unlabeled ligands. Approximately 12 mg of snail hemagglutinin and a concentration of [^{3}H]AR$_L$ 0.52 which gives 110 × 10^{-6} M bound radioactive hapten were employed for each determination. Displacing haptens were AR$_L$ 0.52 (●—●) self-displacement curve; Me-α-D-GalNAc (□—□), Me-α-D-GNac (▲—▲), and Et-β-D-GNAc (○—○). The dashed horizontal line signifies the concentration of bound radioactive hapten in the absence of competitor.

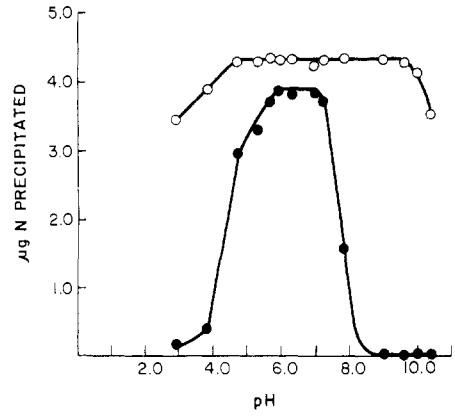

FIGURE 3: Effect of hydrogen ion concentration on precipitation of purified *Helix pomatia* A hemagglutinin by human blood group A substance or by teichoic acid of *S. aureus* strain 3528, respectively. 3.9 μg N of snail hemagglutinin and 9.4 μg of A substance, MSM (○—○), or 3.4 μg of *S. aureus* strain 3528 teichoic acid (●—●), respectively, were used. The total volume was 200 μl. The following buffers were used: pH 2.9–7.1, 0.1 M citrate–phosphate buffer; pH 5.3–7.8, 0.1 M phosphate buffer; pH 9.0–10.4, carbonate–bicarbonate buffer. The sodium chloride concentration was 0.9% throughout.

of combining sites per mole of hemagglutinin (Figure 1A)) and according to the Sips distribution (Sips, 1948; Karush, 1962) (log $(r/(n - r)) = a \log c + a \log K_0$; a is the index of heterogeneity) (Figure 1B). The intercept on the x axis indicates that there are six combining sites per mole of hemagglutinin (Figure 1A). The molecular weight used for calculation of molar hemagglutinin concentration was 1.0×10^5 (Hammarström and Kabat, 1969). The intrinsic association constant, K_0, for the interaction between the hemagglutinin and AR$_L$ 0.52 was 5.0×10^3 l./mole.

The plot of r/c *vs.* r over a wide range of r (0.4–5.3) was linear (Figure 1A) and the Sips distribution function also failed to demonstrate substantial heterogeneity of association constants since the heterogeneity index, a, was 1.04 determined as the slope of the line drawn by the method of least squares (Figure 1B).

Further evidence for homogeneity of combining sites of the hemagglutinin was provided by equilibrium dialysis displacement experiments in which labeled AR$_L$ 0.52 was displaced from the hemagglutinin sites by increasing concentrations of various competitors. The competitors studied were those giving inhibition of precipitation in our earlier study (Hammarström and Kabat, 1969); *i.e.*, Me-α-D-GalNAc, Me-α-D-GNAc, and Et-β-D-GNAc. The data are plotted as concentration of bound labeled hapten *vs.* concentration of free competitor (Figure 2). Also included is the self-displacement curve of AR$_L$ 0.52 calculated as described by Nisonoff and Pressman (1958). All three competitors gave linear displacement curves essentially parallel with that of AR$_L$ 0.52 in this plot indicating that over the range tested (from 10 to 70–80% displacement of labeled AR$_L$ 0.52) there is no substantial heterogeneity of association constants for the binding of the displacing haptens by *H. pomatia* A hemagglutinin.

TABLE I: Intrinsic Association Constants (K_0) and Standard Free-Energy Change ($-\Delta F°$) for the Interaction of *Helix pomatia* A Hemagglutinin with Various Haptens at pH 7.3 and 25.0 ± 0.1°.

Hapten	$K_0 \times 10^{-3}$ (l./mole)	$-\Delta F°$ (kcal/mole)
AR$_L$ 0.52[a]	5.0	5.04
Me-α-D-GalNAc	5.0	5.04
Me-α-D-GNAc	1.1	4.14
Et-β-D-GNAc	0.18	3.08

[a] Blood group A-active reduced pentasaccharide; see Materials and Methods.

Table I gives intrinsic association constants (K_0) and standard free-energy changes ($-\Delta F°$) for the interactions between the hemagglutinin and the above haptens. The association constants for Me-α-D-GalNAc, Me-α-D-GNAc, and Et-β-D-GNAc were calculated from the displacement experiment and K_0 for AR$_L$ 0.52 obtained by direct binding (Nisonoff and Pressman, 1958).

Effect of pH on Precipitation. Figure 3 shows the effect of hydrogen ion concentration on precipitation at equivalence of purified snail hemagglutinin and human blood group A substance or teichoic acid from *S. aureus* strain 3528, respectively. Maximum precipitation of hemagglutinin with blood group A substance occurs over a wide pH range (4.5–9.5). For the reaction with teichoic acid, however, this range is much smaller (5.5–7). In both systems, supernatants from the pH regions of maximum precipitation failed to agglutinate A erythrocytes indicating that precipitation of hemagglutinin was complete.

Inhibition of Precipitation by Various Glycosides. Figure 4A,B shows a precipitation–inhibition experiment in which the inhibiting capacity of phenyl or nitrophenyl α- and β-glycosides of D-GalNAc were compared to the corresponding methyl or ethyl glycosides. Inhibition of precipitation of *H. pomatia* A hemagglutinin and blood group A substance by Ph-α-D-GalNAc and D-GalNAc were the same; *o*- + *p*-

TABLE II: Concentration of D-GalNAc Needed for 50% Inhibition of Precipitation of Purified *Helix pomatia* A Hemagglutinin with Different Carbohydrate Antigens.[a]

Antigen	nmoles of D-GalNAc for 50% Inhibn
Human blood group A substance (cyst MSM; 9.4 μg)	920
Streptococcal group C polysaccharide (4.2 μg)	440
S. aureus strain 3528 teichoic acid (3.4 μg)	11
Ovine submaxillary gland mucin (16.7 μg)	40
Desialized submaxillary gland mucin (3.7 μg)	1100
(5.5 μg)	1300
(7.3 μg)	1350
Human blood group H substance, first stage periodate oxidation and Smith degradation (cyst JS; 14.9 μg)	10

[a] For each inhibition curve 3.9 μg N of purified snail hemagglutinin and the amount of antigen indicated in the table (= equivalence point) were used. For DOSM two additional antigen concentrations corresponding to a slight antibody and antigen excess, respectively, were also studied. Total volume 200 μl.

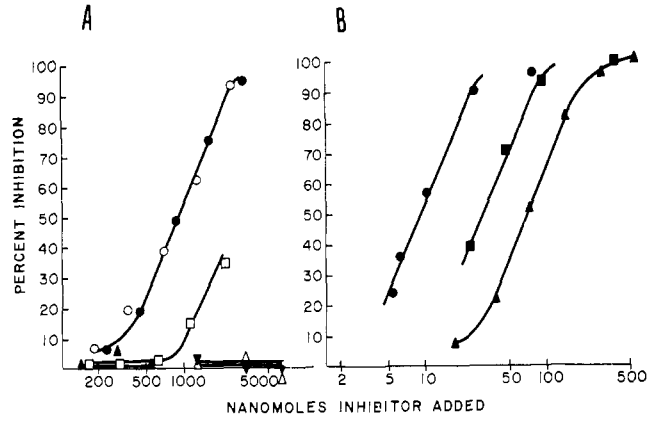

FIGURE 4: Inhibition by methyl glycosides and monosaccharides of precipitation of human blood group A substance (A) or periodate oxidized and Smith degraded human blood group H substance (B) with purified *Helix pomatia* A hemagglutinin. 3.9 μg N of snail hemagglutinin and 9.4 μg of human blood group A substance MSM or 14.9 μg of JS, first periodate stage, were used. The total volume was 200 μl. The following inhibitors were studied: D-GalNAc (●—●), Ph-α-D-GalNAc (○—○), *o*- + *p*-NO_2Ph-α-D-GalNAc (□—□), *p*-NO_2Ph-β-D-GalNAc (▲—▲), Et-β-D-GalNAc (■—■), 2-acetamido-2-deoxy-D-galactitol (▼—▼), 2-acetamido-2-deoxy-D-glucosaminitol (△—△).

Ph-α-D-GalNAc was about one-half as active as D-GalNAc. Me-α-D-GalNAc has previously been shown to be twice as effective as D-GalNAc in this system (Hammarström and Kabat, 1969); thus neither the α-linked phenyl nor the α-linked *o*- + *p*-nitrophenyl group increased the binding to the snail hemagglutinin as compared with an α-linked methyl group on D-GalNAc. The *p*-NO_2Ph-β-D-GalNAc could only be tested up to a concentration of 3×10^{-3} M, due to its low solubility. At this concentration no inhibition was detected (Figure 4A).

For inhibition assays with *p*-NO_2Ph-β-D-GalNAc and Et-β-D-GalNAc, the snail hemagglutinin was precipitated with periodate oxidized Smith degraded human blood group H substance (JS first periodate stage). In this much more sensitive system 50% inhibition with D-GalNAc was achieved with 10 nmoles as compared to 920 nmoles for the *H. pomatia* A hemagglutinin–blood group A substance system. As can be seen *p*-NO_2Ph-β-D-GalNAc was about half as active as Et-β-D-GalNAc, again demonstrating that no increased binding is obtained with the nitrophenyl group.

No inhibition of precipitation was obtained either with *N*-acetyl-D-galactosaminitol or *N*-acetyl-D-glucosaminitol up to concentrations of approximately 6500 nmoles/200 μl. This is about seven times the concentration for 50% inhibition with D-GalNAc and a concentration corresponding to 60% inhibition with D-GNAc.

Precipitation with Glycoproteins and Polysaccharides Containing Nonreducing End Groups of D-GalNAc or D-GNAc. Precipitation between purified snail hemagglutinin and group A, A_{var}, and C polysaccharides (Krause, 1963) is shown in Figure 5A. Complete precipitation at low concentrations was obtained with group C polysaccharide, while group A and group A_{var} streptococcal polysaccharides did not precipitate. Optical rotatory dispersion curves on the three polysaccharides (see Appendix) indicated that D-GalNAc was linked α in group C polysaccharide while D-GNAc was β linked in group A. In the A_{var} the circular dichroism spectrum indicated the D-GNAc to be β linked.

DOSM (sialic acid 0.4%) was completely precipitated at low concentrations and approximately 4.5 times more material was needed for the same degree of precipitation with untreated OSM (Figure 5B).

Teichoic acid from *S. lactis* NCTC 2102, an *N*-acetylglucosamine 1-phosphate polymer with strong positive optical rotation (Archibald *et al.*, 1968), did not precipitate with the hemagglutinin. *S. typhimurium* LT-2 lipopolysaccharide also did not precipitate; this lipopolysaccharide contains terminal α-linked 2-*O*-acetylabequosyl residues (Hellerqvist *et al.*, 1969), which have the same steric orientation as α-linked D-GalNAc of the hydroxyls on carbon-1 and -4 and an equatorial *O*-acetyl group on carbon-2 as compared to an equatorially oriented *N*-acetyl group on the same carbon in D-GalNAc.

Table II gives the concentrations of D-GalNAc needed for 50% inhibition of precipitation of *H. pomatia* A hemagglutinin by various carbohydrate antigens. Inhibition was performed at equivalence and for DOSM in antigen and antibody excess as well. In all systems complete precipitation was obtained at equivalence. Direct comparison of the values should be possible since each system, except for the reaction with periodate-treated blood group H substance, involves the same amount of snail hemagglutinin and approximately the same number of reactive end groups of the antigens as calculated from chemical composition. For periodate-treated blood group H substance there is little information as to the chemical nature of the reactive end groups. As can be seen from the table the concentration of D-GalNAc needed for inhibition in different systems varied over a 100-fold range.

Discussion

The present investigation provides additional information about the combining site of purified *H. pomatia* A hemagglu-

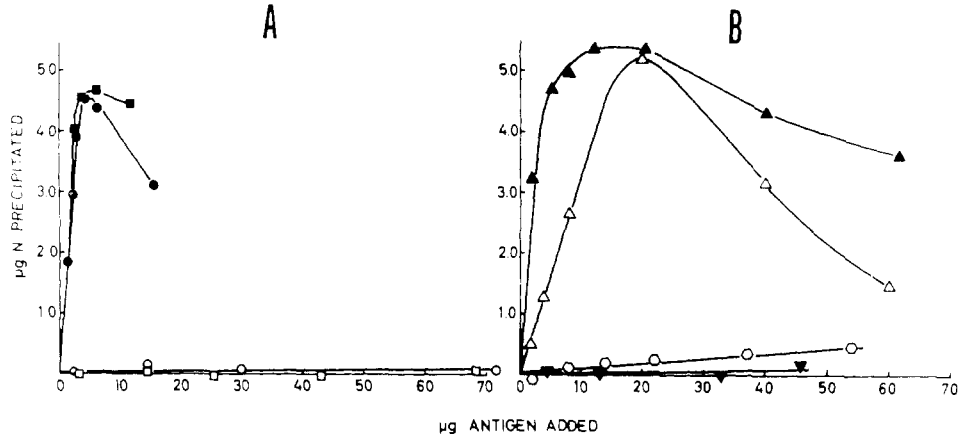

FIGURE 5: Precipitation studies with purified *Helix pomatia* A hemagglutinin and various carbohydrate antigens. (A) 3.9 μg N of snail hemagglutinin precipitated with streptococcal group C polysaccharide, preparation I (●—●) and preparation II (■—■), and with streptococcal group A (○—○) and group A var (□—□) polysaccharides. (B) Same amount of snail protein precipitated with ovine submaxillary mucin (△—△), desialized ovine submaxillary mucin (▲—▲), *S. typhimurium* strain LT-2 lipopolysaccharide (▼—▼), and poly-(GNAc-1 phosphate) from *S. lactis* NCTC 2102 (○—○). The total volume was 200 μl.

tinin. Equilibrium dialysis with the A-active reduced pentasaccharide [^{3}H]AR_L 0.52, an excellent inhibitor of precipitation of hemagglutinin by A substance, showed the hemagglutinin to have six combining sites of intrinsic association constant of 5.0×10^3 l./mole at 25.0°; within experimental error all combining sites bound the hapten equally. Site homogeneity was also indicated by displacement experiments with other related haptens since the ratio between free concentration of the labeled and displacing hapten was constant over the range tested (Figure 2). Nisonoff and Pressman (1958) demonstrated that parallel displacement curves in the plot are only obtained with a homogeneous population of sites or with a heterogeneous population in which the relative association constants of different haptens are the same for each kind of site. However, since site homogeneity could be demonstrated toward the first hapten ([^{3}H]AR_L 0.52), the second case does not apply.

These results are in contrast to hapten binding data obtained with most antibody populations with animals immunized with proteins, hapten–protein conjugates, or with carbohydrates. These show substantial heterogeneity. However, recently, antibody populations with no or very restricted site heterogeneity have been obtained after immunization with pneumococcal vaccine (Katz and Pappenheimer, 1969), with streptococci (Eichmann *et al.*, 1970), or with the peptide hormone, vasopressin (Wu and Rockey, 1969). Similarly the plant hemagglutinin, concanavalin A, showed limited association constant heterogeneity when tested against both Me-α-D-Man and Me-α-D-Glc (So and Goldstein, 1968).

It is of interest that the association constants for the interaction between *H. pomatia* A hemagglutinin and the best inhibitors, AR_L 0.52 or methyl α-D-GalNAc, are of the same order of magnitude as for other carbohydrate–anti-carbohydrate systems, notably human anti-A antibodies from three individuals and AR_L 0.52, $K_0 = 2 \times 10^3$ to 1×10^5 l./mole at 25° (Moreno and Kabat, 1969); rabbit anti-*p*-azophenyl β-lactoside antibodies and *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside, $K_0 = 1.3 \times 10^5$ l./mole at 25° (Karush, 1958); and rabbit anti-pneumococcal type III antibodies and hexasaccharides from S III, $K_0 = 1.4$–3.2×10^5 l./mole at 4° (Katz and Pappenheimer, 1969). K_0 for the interaction between concanavalin A and the best inhibitor Me-α-D-Man is also similar, 2×10^4 l./mole at 2° (So and Goldstein, 1968).

In earlier studies (Hammarström and Kabat, 1969) the specificity of the hemagglutinin site as defined by the ability of various haptens to inhibit precipitation between blood group A substance and the hemagglutinin was investigated in some detail. These experiments demonstrated that Me-α-D-GalNAc was the best inhibitor and that roughly 2, 4.5, and 10 times higher concentrations of AR_L 0.52, Me-α-D-GNAc, and Et-β-D-GNAc, respectively, were needed for comparable degrees of inhibition. From equilibrium dialysis displacement experiments the relation between the four inhibitors was 1, 1, 4.5, and 27; thus there is only fair agreement between these two types of measurements. This has also been found by others (Nisonoff and Pressman, 1958). However, in their studies, heterogeneity of combining sites is a further complication. It is reasonable to assume that the replacement experiments give a more accurate definition of the site than inhibition of precipitation since the former system is a simple equilibrium reaction.

Ph- (or NO_2Ph-) α- or β-D-GalNAc inhibited precipitation between the hemagglutinin and blood group A substance (or periodate-treated H substance) to a somewhat lesser extent than did Me- (Et-) α- and β-D-GalNAc, respectively. This is in contrast to the findings for concanavalin A (Goldstein and Iyer, 1966; Poretz and Goldstein, 1970). Ph-β-D-Glc (or NO_2Ph-β-D-Glc) inhibited precipitation of concanavalin A with levan at approximately one-fifth the concentration needed for Me- (or Et-) β-D-Glc (Poretz and Goldstein, 1970).

There was no inhibition with either *N*-acetylgalactosaminitol or *N*-acetylglucosaminitol, indicating that the pyranoside ring is important for the proper orientation of the hydroxyl groups and the acetamido group in reacting with the site.

Earlier studies (Hammarström and Kabat, 1969) demonstrated that Et-β-D-GalNAc did not inhibit precipitation of the hemagglutinin by human blood group A substance up to a concentration of approximately 4300 nmoles/200 μl (about 2.6 times the concentration needed for 50% inhibition with D-GalNAc). However, when inhibition with Et-β-D-GalNAc was investigated in the system, blood group H substance first IO_4 stage–*H. pomatia* A hemagglutinin, this inhibitor

was relatively potent (50% inhibition with 3.3 times the concentration needed for D-GalNAc). The reason for this discrepancy is not known.

Further information on the specificity of the hemagglutinin site was obtained from precipitation analysis with different carbohydrate antigens. The lack of precipitation between *H. pomatia* A hemagglutinin and *S. typhimurium* strain LT-2 lipopolysaccharide (Figure 5B) indicates that the hydroxyl groups on either carbon-3, -6, or both are of importance for binding to the site. Thus proper steric orientation of the substituents on C-1, C-2, and C-4 is not sufficient for reactivity. An *O*-acetyl group can be equated with an *N*-acetyl group in this system since Me-α-D-GalNAc gives the same degree of inhibition as the methyl α-glycoside of 2-*O*-Ac-D-Gal (S. Hammarström, unpublished observations).

No precipitation was obtained when teichoic acid from *S. lactis* NCTC 2102 was reacted with the hemagglutinin (Figure 5B), indicating that α-linked D-GNAc in a 1→6 linear polymer is nonreactive.

When *H. pomatia* A hemagglutinin was tested against streptococcal polysaccharides from group A, A_{var}, and C, respectively, complete precipitation was obtained with group C while group A and A_{var} were negative. Structural analysis has shown that the group A and A_{var} consist of a rhamnosyl-(1→3)-rhamnose backbone, probably α linked, substituted with multiple side chains composed of GNAc-Rha or Rha, respectively (McCarty, 1956; Heymann *et al.*, 1963; Krause, 1963). Enzymatic studies by McCarty (1956) demonstrated that much if not all of the terminal nonreducing D-GNAc is β linked. Optical rotatory dispersion and circular dichroism measurements confirm this (see Appendix). No precipitation was seen with the hemagglutinin and the streptococcal A polysaccharide in accord with the findings with teichoic acids from *S. aureus*, containing β-linked D-GNAc nonreducing end groups which also did not precipitate (Hammarström and Kabat, 1969). It is also in accord with the weak interaction of Et-β-D-GNAc with the hemagglutinin site ($K_0 = 0.18 \times 10^3$ l./mole, at 25°).

In the studies with teichoic acids it was found that the α-D-GNAc polymer was completely precipitated with the hemagglutinin. K_0 for the interaction of the hemagglutinin and Me-α-D-GNAc was 1.1×10^3 l./mole at 25°. Since the β-linked group A streptococcal polysaccharide did not precipitate with the hemagglutinin, and assuming that both polysaccharides have equal numbers of reactive D-GNAc residues per molecule, the lower limit of binding strength needed for precipitation in this system might correspond to an intrinsic association constant of between 1000 and 200 l. per mole at 25°.

Chemical and immunological data indicate that group C and A streptococcal polysaccharides have the same general structure except that D-GalNAc is substituted for D-GNAc (Krause, 1963; Karakawa *et al.*, 1965). There is good indirect evidence that the linkage of terminal GalNAc to Rha may be α (Krause, 1963; Michel *et al.*, 1969; Etzler and Kabat, 1970). The optical rotatory dispersion and circular dichroism spectra on the C-polysaccharide strongly indicate the presence of an α linkage (see Appendix).

Chemical studies on OSM have shown that most of the carbohydrate was accounted for by sialyl-*N*-acetylgalactosamine disaccharide moieties (Pigman and Gottschalk, 1966), which are bound by *O*-glycosidic linkage to serine and threonine in the peptide core (Anderson *et al.*, 1964; Bertolini and Pigman, 1967). Enzymatic studies by Weissmann and Hinrichsen (1969) using mammalian α-*N*-acetyl-D-galactosaminidase have conclusively demonstrated that D-GalNAc is α-glycosidically linked to the peptide. Our findings that OSM and DOSM precipitate well with the hemagglutinin (Figure 5B) are consistent with the chemical data and indicate that approximately one-fifth of the D-GalNAc residues in the OSM preparation are not substituted with *N*-acetylneuraminic acid, a value in good accord with 0.85 for its NANA:GalNAc ratio; similar ratios have been found by others (Weissmann and Hinrichsen, 1969).

The utility of reagents like the *H. pomatia* A hemagglutinin for the detection of specific groups on macromolecules depends on a detailed knowledge of their specificity as well as on their behavior in the assay systems. The influence of hydrogen ion concentration on the precipitin reaction between the hemagglutinin and two polysaccharides containing different reactive groups is illustrative (Figure 3). When the hemagglutinin reacts with blood group A substance, α-linked-D-GalNAc end groups have previously been shown to be responsible for the major reactivity (Hammarström and Kabat, 1969). In this system changes in pH between 4 and 9.5 are of no influence; only rather extreme hydrogen ion concentrations abolish precipitation. However, when the hemagglutinin reacts with α-linked D-GNAc end groups, as with teichoic acids, pH becomes of much greater importance, complete precipitation only being obtained between 5.5 and 7. The major difference between these two systems, which involve the same amount of *H. pomatia* A hemagglutinin sites and approximately the same number of end groups in each substance, is probably the difference in association constants for the two interactions (roughly 4.5-fold). The difference between the two systems was also evident when borate was used to dissociate the precipitate (Svensson *et al.*, 1970). Moreover, when D-GalNAc was used for inhibition, roughly 100 times more inhibitor was needed to inhibit the α-D-GalNAc system than the α-D-GNAc system (Table II).

From Table II it can also be seen that precipitation by other carbohydrate antigens with α-linked D-GalNAc nonreducing end groups (*e.g.*, streptococcal group C polysaccharide and DOSM) is roughly as difficult to inhibit with D-GalNAc as precipitation by blood group A substance. This finding supports the notion that the strength of binding of the hemagglutinin site for the reactive group in the glycoprotein or polysaccharide is an important factor in determining the concentration of a given inhibitor needed. Similar observations have been made (So and Goldstein, 1968) for the reaction of concanavalin A with levan, with dextran, and with α-mannan, respectively. The effectiveness of inhibition of the sugars constituting the reactive end groups in the three polysaccharides was Me-α-D-Man > Me-α-D-Glc > methyl β-D-fructofuranoside. Moreover, the concentration of Me-α-D-Glc for a given degree of inhibition was greatest for the reaction of concanavalin A with mannan, less with dextran, and least with levan, respectively.

However, the degree of inhibition of precipitation of *H. pomatia* A hemagglutinin by various glycoproteins or polysaccharides may be affected by such factors as charge of the macromolecule, concentration of reactive groups, etc. Thus, when inhibition of precipitation by D-GalNAc was compared in the reaction of DOSM and OSM with snail hemagglutinin (Table II) a very marked difference in the concentration needed for 50% inhibition was obtained (1300 and 40 nmoles, respectively). Since this comparison involves the same reactive group in the glycoproteins and approximately the same number of hemagglutinin sites,

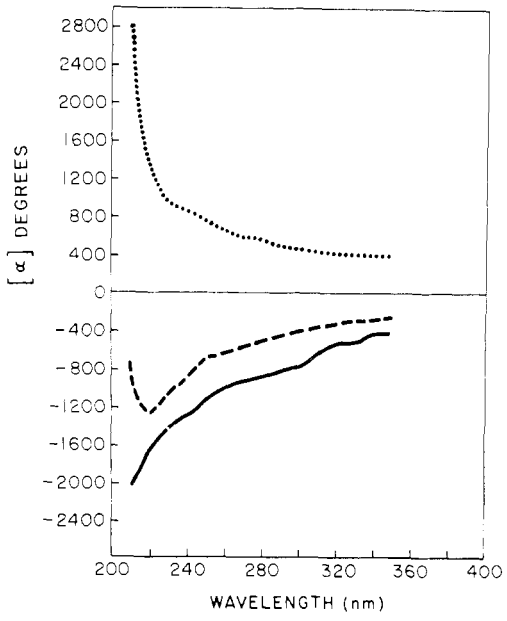

FIGURE 6: Optical rotatory dispersion spectra of groups A, A_{var}, and C streptococcal polysaccharides. ....., group C; ———, group A_{var}; — — —, group A. Measurements made in 0.50-cm cell at ambient temperature. Concentrations: group C, 0.775 mg/ml; group A_{var}, 1.46 mg/ml; group A, 0.725 mg/ml.

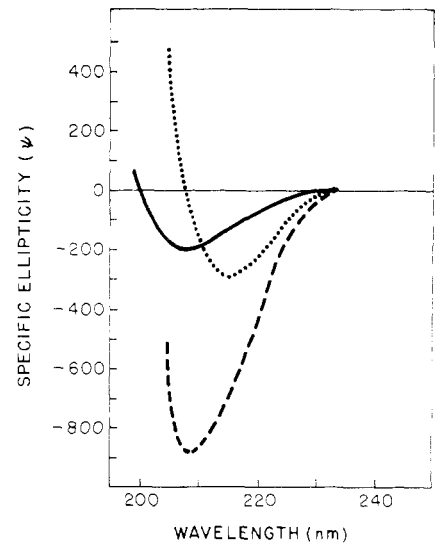

FIGURE 7: Circular dichroism spectra of groups A, A_{var}, and C streptococcal polysaccharides. ....., group C; ———, group A_{var}; — — —, group A. Concentrations and path length same as in Figure 6. The ordinate is explained in the text.

the result clearly demonstrates the role of other factors. It is probable that the negatively charged NANA groups surrounding the α-linked D-GalNAc groups on neighboring chains repel the hemagglutinin. In a study by Goldstein *et al.* (1968) inhibition by Me-α-D-Glc was compared in the concanavalin A–dextran system using dextrans with different degree of branching. It was found that highly branched dextran was more difficult to inhibit than less branched dextran. It should be noted, however, that precipitation was incomplete with dextran containing few branches.

Thus, charge of the macromolecule and concentration of reactive groups have to be considered when inhibition of precipitation is used to detect reactive end groups in unknown substances.

Appendix: Analysis of Optical Rotatory Dispersion and Circular Dichroism Spectra of Groups A, A_{var}, and C Streptococcal Polysaccharides

By Sherman Beychok, Sten Hammarström, and Elvin A. Kabat

The optical rotatory dispersion spectra of streptococcal polysaccharides from groups A, A_{var}, and C are shown in Figure 6. The circular dichroic spectra of these polysaccharides are shown in Figure 7. In previous reports, it has been shown that the optical rotatory dispersion and circular dichroism spectra of oligo- and polysaccharides which contain D-GNAc and D-GalNAc residues can be used to infer the nature of the glycosidic linkage (α or β) as well as other structural features involving these residues (Beychok and Kabat, 1965; Lloyd *et al.*, 1967, 1968; Kabat *et al.*, 1969). In order to do so, however, it is necessary to know the hexosamine content and the average residue molecular weight. The approximate hexosamine composition of these polysaccharides is given in Table III. Because of uncertainty in these values, the figures present the results in terms of specific rotations and ellipticities, rather than corresponding molar quantities. Thus, the ordinates are based on observed values and calculated as in eq 1 and 2.

$$[\alpha] = \frac{\alpha_{obsd} \times 100}{dc} \quad (1)$$

$$[\psi] = \frac{\theta_{obsd} \times 100}{dc} \quad (2)$$

The ellipticities are thus expressed in the same dimensions as the more familiar specific rotation. In these equations, α_{obsd} and θ_{obsd} are the measured rotation and ellipticity, d is the path length in decimeters, and c the concentration in g/100 ml.

These quantities may be converted to the more useful molar expressions 3 and 4, in which $[m]$ and $[\theta]$ are the

$$[m] = \frac{[\alpha] \times MW}{100} \quad (3)$$

$$[\theta] = \frac{[\psi] \times MW}{100} \quad (4)$$

molar rotation and ellipticity, respectively, and MW is the molecular or residue weight. To infer the contributions to these spectra of the D-GNAc and D-GalNAc residues for the purpose of deciding the nature of the glycosidic linkages, the molar quantities of eq 3 and 4 are required. In Table III, the relevant parameters are given, and computations using eq 3 and 4 may be made, recognizing the approximate nature of the analytical values for D-GNAc and D-GalNAc content.

Before discussing these, certain qualitative features in Figures 6 and 7 may be examined. In the optical rotatory dispersion spectra, only the curve for the polysaccharide of group A exhibits a distinct trough of a negative Cotton effect. This trough is associated with β-linked D-GNAc residues, the only other constituents in the polysaccharide being L-

TABLE III: Optical Rotatory Dispersion and Circular Dichroism Parameters of Streptococcal Polysaccharides of Group A_{var}, Group A, and Group C.

Substance	Approx Hexosamine Content (%)	Approx Mean Residue Wt	$m_{220} - m_{300}$ Av Residue Wt Basis	$m_{220} - m_{300}$ per Mole of D-GNAc or D-GalNAc	[θ]/Mole of D-GNAc or D-GalNAc
Group A_{var}	4	148	−1300		−7800
Group A	27	160	−1390	−2000[a]	−5350
Group C	33	163	1480	7520[a]	−1500
Me-α-D-GNAc				1930	−4000
Me-β-D-GNAc				−2650	−6300
Me-α-D-GalNAc				3300	−3000
Me-β-D-GalNAc				−470	−4600

[a] These values are computed by assuming that $m_{220} - m_{300}$ generated by L-rhamnosyl residues is the same in the polysaccharides of group A and C as the value given in column 4 for group A_{var}. The streptococcal polysaccharides were supplied by Drs. R. M. Krause and M. McCarty; references are given in the main paper.

rhamnosyl residues, which should not generate a Cotton effect in this wavelength interval (Listowsky *et al.*, 1965). The optical rotatory dispersion spectrum of the polysaccharide of group A_{var} is negative in the wavelength interval shown, but exhibits no obvious Cotton effect. The reason for this is the low D-GNAc content (Table III). It is readily calculated that the D-GNAc residues, even if all β linked and internal, could not contribute enough to the spectrum to overcome the steeply falling background of the L-rhamnosyl residues. Indeed, the spectrum may be taken approximately to be that of the L-rhamnosyl residues in this polysaccharide. The optical rotatory dispersion spectrum of the polysaccharide of group C is everywhere positive, but again, no Cotton effect is discerned. However, the positive D-GalNAc contribution is large enough to overcome the negative curve generated by the L-rhamnosyl residues. Qualitatively, this implies that the predominant linkage of the D-GalNAc residues is α.

The circular dichroism spectra of the three polysaccharides (Figure 7) reveal the D-GNAc and D-GalNAc bands. Because the L-rhamnosyl residues make no contribution to background in these spectra, it is possible to discern even the small mole fraction of D-GNAc residues in the group A_{var} spectrum. At the same time, to evaluate, from the data, the nature of the linkage requires computation of the molar ellipticities on the basis of the content of D-GNAc and D-GalNAc residues. Results of such computations are tabulated in Table III. The uncertainty of the computed values resides in the uncertainty of hexosamine content. The values for the latter in the table are subject to an error of about 10% and may often be low especially because of the stabilization of the α-glycoside linkage by the positive charge of a hydrolyzed *N*-acetyl group (Shively and Conrad, 1970). It has been noted previously that values of $m_{220} - m_{300}$ for oligosaccharides of known composition allow decisions about the nature of the linkage or residues containing the 2-deoxy-2-acetamido group and, in addition, that substituted D-GNAc or D-GalNAc residues give values of $m_{220} - m_{300}$ which fall into different ranges depending on the position and nature of the substituent (Kabat *et al.*, 1969). Estimates of values of $m_{220} - m_{300}$ for the three polysaccharides are given in column 5 of Table III. Values of $m_{220} - m_{300}$ for the methyl glycosides of D-GNAc and D-GalNAc are included for comparison. The calculation *assumes* that the L-rhamnosyl background in the spectra of the group A and group C polysaccharides is approximated by the optical rotatory dispersion curve of the group A_{var}. The mole fraction of D-GNAc residues is only about 0.036 in this polysaccharide and it can be calculated that the contributions of such residues to the value of $m_{220} - m_{300}$ is less than −100 deg cm²/dmole. With this assumption, one can calculate approximate molar difference values for the other two polysaccharides. In the case of group A polysaccharide, the value is quite close to that of Me-β-D-GNAc, and of different sign from that of Me-α-D-GNAc. Within the limits of approximation, it is not possible to state that only β-linked D-GNAc residues occur, but such linkages are clearly predominant. The group C polysaccharide gives a positive value twice as great as that of Me-α-D-GalNAc. β-Linked residues seem out of the question. The discrepancy between the polysaccharide and Me-α-D-GalNAc appears too large to be ascribed to the uncertainties of the estimate. In part, the difficulties of hydrolysis of α-linked GalNAc residues (*cf.* above) may contribute. Moreover, the assumption that the L-rhamnosyl residues make comparable background contributions in A_{var} and C may be poor. With the large number of D-GalNAc groups involved, every other L-rhamnosyl residue is disubstituted, on the average, with a D-GalNAc residue and an L-rhamnosyl residue. Still, the good agreement with the group A polysaccharide leaves this question in doubt.

The circular dichroism results tend to enforce these results. There is no need to make any assumption about the L-rhamnosyl background, since it is absent or small. For the A_{var} polysaccharide, the value is higher than for Me-β-D-GNAc. This may be due to uncertainty in the computation, since a 10% error in hexosamine content would have an important effect. Alternatively, the high value may be the result of the fact that in A_{var} the β-linked D-GNAc residues are internal and thus substituted at positions 2, 3, or 6. In each case, the [θ] value would be increased in magnitude, on the basis of observations with other compounds (Kabat *et al.*, 1969). However, there is little doubt that the GNAc residues are β linked.

The polysaccharide from group C is again, by circular dichroism, somewhat anomalous. The value is too low compared to Me-α-D-GalNAc and much too low in comparison to the Me-β-D-GalNAc. Thus, there is no reason to

expect any β linkages, but in agreement with the optical rotatory dispersion findings some structural feature is adding a positive contribution, or diminishing the negative contribution expected for α-linked D-GalNAc residues.

The group A polysaccharide gives a value intermediate between β- and α-linked D-GNAc residues. Here, too, uncertainty in composition is important. If there are no internal residues, the result would indicate that not all of the terminal D-GNAc are β linked. Taken together with the optical rotatory dispersion result, it might be concluded that terminal β-linked D-GNAc residues are predominant but a small proportion of α linkages cannot be excluded.

It should be noted that the circular dichroism extrema in Figure 7 differ for the various polysaccharides. Thus in groups A_{var} and A, they are at 208 and 209 nm while in group C, the extremum is at 215 nm. The extrema in circular dichroism spectra as well as in optical rotatory dispersion spectra have differed for the various oligosaccharides studied. Data are insufficient to provide a satisfactory explanation for the variations in wavelengths. Attempts are under way to examine such differences for a large number of compounds to evaluate its structural significance.

References

Anderson, B., Seno, N., Sampson, P., Riley, J. G., Hoffman, P., and Meyer, K. (1964), *J. Biol. Chem. 239*, PC2716.

Archibald, A. R., Baddiley, J., Button, D., Heptinstall, S., and Stafford, G. H. (1968), *Nature (London) 219*, 855.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Bertolini, M., and Pigman, W. (1967), *J. Biol. Chem. 242*, 3776.

Beychok, S., and Kabat, E. A. (1965), *Biochemistry 4*, 2565.

Bird, G W. G. (1959), *Brit. Med. Bull. 15*, 165.

Eichmann, K., Lackland, H., Hood, L., and Krause, R. M. (1970), *J. Exp. Med. 131*, 207.

Etzler, M., and Kabat, E. A. (1970), *Biochemistry 9*, 869.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Goldstein, I. J., Hay, G. W., Lewis, B. A., and Smith, G. (1965), *Methods Carbohyd. Chem. 5*, 361.

Goldstein, I. J., and Iyer, R. N. (1966), *Biochim. Biophys. Acta 121*, 197.

Goldstein, I. J., Poretz, R. D., So, L. L., and Yang, Y. (1968), *Arch. Biochem. Biophys. 127*, 787.

Hammarström, S., and Kabat, E. A. (1969), *Biochemistry 8*, 2696.

Hellerqvist, C. G., Lindberg, B., Svensson, S., Holme, T., and Lindberg, A. A. (1969), *Carbohyd. Res. 9*, 237.

Heymann, H., Manniello, J. M., and Barkulis, S. S. (1963), *J. Biol. Chem. 238*, 502.

Kabat, E. A. (1956), Blood Group Substances: Their Chemistry and Immunochemistry, New York, N. Y., Academic Press.

Kabat, E. A. (1961), Kabat and Mayer's Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas.

Kabat, E. A., Lloyd, K. O., and Beychok, S. (1969), *Biochemistry 8*, 747.

Karakawa, W. W., Krause, R. M., and Borman, E. K. (1965), *J. Immunol. 94*, 282.

Karush, F. (1958), *Trans. N. Y. Acad. Sci. 20*, 581.

Karush, F. (1962), *Advan. Immunol. 2*, 1.

Katz, M., and Pappenheimer, Jr., A. M. (1969), *J. Immunol. 103*, 491.

Krause, R. M. (1963), *Bacteriol. Rev. 27*, 369.

Krause, R. M., and McCarty, M. (1962), *J. Exp. Med. 115*, 49.

Listowsky, I., Avigad, G., and Englard, S. (1965), *J. Amer. Chem. Soc. 87*, 1765.

Lloyd, K. O., Beychok, S., and Kabat, E. A. (1967), *Biochemistry 6*, 1448.

Lloyd, K. O., Beychok, S., and Kabat, E. A. (1968), *Biochemistry 7*, 3762.

Lloyd, K. O., and Kabat, E. A. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1470.

Lloyd, K. O., Kabat, E. A., Layug, E. J., and Gruezo, F. (1966), *Biochemistry 5*, 1489.

Ludowieg, J., and Benmaman, J. D. (1967), *Anal. Biochem. 19*, 80.

Mäkelä, O. (1957), *Ann. Med. Exp. Biol. Fenn. 35, Suppl. 11.*

McCarty, M. (1956), *J. Exp. Med. 104*, 629.

Michel, M. F., van Vonno, J., and Krause, R. M. (1969), *J. Immunol. 102*, 215.

Moreno, C., and Kabat, E. A. (1969), *J. Exp. Med. 129*, 871.

Nathenson, S. G., Ishimoto, N., Anderson, J. S., and Strominger, J. L. (1966), *J. Biol. Chem. 241*, 651.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 81*, 126.

Pigman, W., and Gottschalk, A. (1966), *in* Glycoproteins, Gottschalk, A., Ed., Amsterdam, Elsevier, p 434.

Poretz, R. D., and Goldstein, I. J. (1970), *Biochemistry 9*, 2890.

Prokop, O., Rackwitz, A., and Schlesinger, D. (1965a), *J. Forensic. Med. 12*, 108.

Prokop, O., Schlesinger, D., and Rackwitz, A. (1965b), *Z. Immunitatsforsch. Allerg. Klin. Immunol. 129*, 402.

Prokop, O., Uhlenbruck, G., and Kohler, W. (1968), *Vox. Sanguinis, 14*, 321.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Schiffman, G., Kabat, E. A., and Thompson, W. (1964), *Biochemistry 3*, 113.

Shively, J. E., and Conrad, H. E. (1970), *Biochemistry 9*, 33.

Sips, R. (1948), *J. Chem. Phys. 16*, 490.

So, L. L., and Goldstein, I. J. (1968), *J. Biol. Chem. 243*, 2003.

Svensson, S., Hammarström, S. G., and Kabat, E. A. (1970), *Immunochemistry 7*, 413.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Weir, D. M. (1967), Handbook of Experimental Immunology, 1st ed, Oxford and Edinburgh, Blackwell, p 493.

Weissmann, B., and Hinrichsen, D. F. (1969), *Biochemistry 8*, 2034.

Wu, W. H., and Rockey, J. H. (1969), *Biochemistry 8*, 2719.