Purification and Characterization of a Blood-Group A Reactive Hemagglutinin from the Snail *Helix pomatia* and a Study of Its Combining Site*

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ABSTRACT: A protein specifically agglutinating human A erythrocytes has been isolated from the albumin gland of the snail *Helix pomatia*. The hemagglutinin, obtained in high yield (85.5%) by adsorption to insoluble polyleucyl blood-group A substance and subsequent elution with 0.005 M D-GalNAc, was homogeneous by gel filtration and ultracentrifugation and gave one precipitin band in double diffusion and immunoelectrophoresis against rabbit antisera to the crude extract. It was completely precipitated by human blood-group A substance. On polyacrylamide gel at alkaline pH, several bands with closely similar mobilities were observed, probably ascribable to heterogeneity of hemagglutinin molecules. The amino acid composition has been determined. It contains both half-cystine and methionine and has about 7% carbohydrate of which 4.0% is galactose and 3.3% is mannose. The molecular weight is 100,000. Precipitation between blood-group A substance and purified hemagglutinin is best inhibited with Me-α-D-GalNAc and with twice the molar amount of D-GalNAc. No inhibition was obtained with Et-β-D-GalNAc even at very high concentrations. Me-α-D-GNAc and Me-β-D-GNAc also inhibited precipitation although four and ten times higher concentrations than for Me-α-D-GalNAc were needed. The size of the combining site may be no larger than that of an α-linked monosaccharide since about the same degree of inhibition was obtained by the A-active di-, tri-, and pentasaccharides, isolated from human or hog blood-group A substances, as with Me-α-D-GalNAc. The hemagglutinin not only precipitates with human blood-group A substance but reacts to a lesser extent with B, H, or Le^a blood-group substances and increased reactivity is observed with a P-1 fraction or with a cyst material of low fucose content. The structure responsible for this reaction is not known. Precipitin analyses with teichoic acids from different strains of *Staphylococcus aureus* containing D-GNAc linked either α or β to the ribitol phosphate backbone or with mixtures of the α- and β-D-GNAc polymers revealed that only the α-D-GNAc polymer precipitates; using the 100% α-linked teichoic acid as a standard, the amount of α-linked polymer in mixtures could be estimated quantitatively.

By comparing the relative capacities of Me-α-D-GalNAc, D-GalNAc, Me-α-D-GNAc, and D-GNAc in inhibiting precipitation between blood-group A substance and various fractions of snail hemagglutinin, homogeneity of the combining site was indicated.

Hemagglutinins specific for human A, B, and O erythrocytes occur in vertebrates, invertebrates, and plants (Boyd, 1966; Kabat, 1956; Prokop and Uhlenbruck, 1963; Prokop *et al.*, 1968). Invertebrate agglutinins were discovered most recently (Prokop *et al.*, 1965a,b; Boyd and Brown, 1965) and those from the class Gastropoda (phylum Mollusca) have been most extensively studied (Prokop *et al.*, 1968). Extracts from 19 of 26 different species of snails contained hemagglutinins reacting either with human A or B erythrocytes or with human erythrocytes irrespective of A, B, or O type (Krüpe and Pieper, 1966). The agglutinins were obtained from the albumin gland, which is a part of the sexual apparatus, and also from the eggs (Prokop *et al.*, 1968). Agglutinins of similar specificities have also been found in extracts of fish eggs (Prokop *et al.*, 1968). Their physiological function is not known.

Human antibodies specific for the blood-group A determinant have been shown to be heterogeneous both with respect to size of combining sites (Kaplan and Kabat, 1966; Moreno and Kabat, 1969), heavy and light chains, and genetic markers (Yount *et al.*, 1968; Dorner *et al.*, 1969). Partial fractionation of antibodies with respect to size of combining site can be accomplished by specific elution with haptens representing the entire antigenic determinant or smaller parts of it (Schlossman and Kabat, 1962; Gelzer and Kabat, 1964a,b; Moreno and Kabat, 1969). Antibody heterogeneity seriously limits the study of sequence and the three-dimensional arrangements of amino acids in the combining region. For comparison with human anti-A antibodies the hemagglutinin from the snail *Helix pomatia*, which reacts with blood-group A substance has been purified and characterized. This protein does not show any heterogeneity of combining site and has a relatively small size site.

Materials

H. pomatia was collected outside of Stockholm. The albumin glands from approximately 200 snails were dissected, homogenized in a blendor, and extracted with 0.15 M phosphate-buffered saline (pH 7.2) for 20 min at 4° (300 ml of 0.15 M phosphate-buffered saline was added to 150 g of albu-

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TABLE I: Analytical Data on Purified Blood-Group Substances from Human Ovarian Cysts and Hog Gastric Mucin.

Blood-Group Substance	Blood-Group Specificity	N	Per Cent Composition				
			Fuc	Gal	Hexosamine	$GalNH_2$	GNH_2[a]
Cyst-9[b] C_6H_5OH insoluble	A_1	5.1	20.7	23.5	32.6	20.0	12.6
Cyst-MSM[b] 10% precipitate	A_1	5.0	15.3	22.5	32.0	14.6	17.4
Cyst-MSS[b] 10% precipitate	A_1	5.4	15.5	24.2	31.5	18.8	12.7
Cyst-14[b] 10% precipitate	A_2	6.2	14.7	23.5	23.1	7.0	16.1
Cyst-Beach[b] C_6H_5OH insoluble	B	4.5	19.4	34.7	24.5	8.2	16.3
Cyst-19[b] C_6H_5OH insoluble	B	5.0	17.7	34.5	26.0	7.9	18.1
Cyst-Tighe[b] C_6H_5OH insoluble	O	4.1	26.0	28.9	24.5	7.8	16.7
Cyst-JS[b] C_6H_5OH insoluble	O	4.6	19.3	28.0	25.5	8.3	17.1
Cyst-N-1-1[b] 10% precipitate	Le[a]	4.2	8.5	32.3	31.5	9.0	22.5
Cyst-OG-3[c] 10–20% precipitate	—	6.8	2.4	32.5	29.0	10.3	18.7
Cyst-Beach P-1	—	6.3	5.1	30.4	27.2	14.1	13.1
Hog gastric mucin	A + H	5.3	9.0	20.0	30.7	10.4	20.3

[a] Determined by difference. [b] Lloyd and Kabat, 1968, and earlier papers from this laboratory. [c] Vicari and Kabat (1969).

min gland wet weight). The extract was centrifuged at 12,000 rpm for 20 min at 4° and the precipitate was reextracted with buffered saline. The combined supernatants were then centrifuged for 2.5 hr at 40,000 rpm which resulted in three fractions; a clear light green supernatant, a light precipitate, and a heavy gelatinous precipitate. All three fractions agglutinated human A but not B or O erythrocytes; the supernatant contained significantly higher concentrations of hemagglutinin and was used for further study. About 8% of the nitrogen in this fraction was specifically precipitated with human blood-group A substance. When tested in immunoelectrophoresis against hog gastric mucin, two precipitin arcs were formed. The material was stored at −20° and transported to the U. S. frozen.

The blood-group substances used were the previously described preparations from human ovarian cysts or from hog gastric mucin (*cf.* Kabat, 1956; Schiffman *et al.*, 1964a; Lloyd *et al.*, 1968; Vicari and Kabat, 1969). Cyst Beach P-1 was the nondialyzable fraction obtained after mild acid hydrolysis of cyst Beach as earlier described (Allen and Kabat, 1959). The actual preparations used their analytical figures and blood-group specificities are given in Table I.

Teichoic acids from *Staphylococcus aureus* strains Copenhagen, A1, 3528, and NYH-6 were gifts from Drs. J. L. Strominger, J. Baddiley, J. L. Strominger, D. J. Tipper, and S. I. Morse, respectively. Teichoic acid from *Staphylococcus albus* was a gift from Dr. S. I. Morse. Strain A1 has previously been shown to contain only β-linked GNAc (Davidson *et al.*, 1964), strain 3528 only α-linked GNAc (Nathenson and Strominger, 1962; Nathenson *et al.*, 1966), while the Copenhagen strain is a mixture of approximately 15% α-GNAc-polymer and 85% β-GNAc-polymer (Sanderson *et al.*, 1962; Torii *et al.*, 1964; Nathenson *et al.*, 1966). The NYH-6 strain (Morse, 1962) was considered to contain approximately 50% each of the α- and β-linked GNAc-polymers (Torii *et al.*, 1964; Kabat *et al.*, 1969). *S. albus* strain Prengel is a glycerophosphate polymer containing glucose instead of GNAc (Morse, 1963).

Oligosaccharides were obtained either by mild acid hydrolysis (Schiffman *et al.*, 1962) or by alkaline cleavage in the presence of sodium borohydride (Schiffman *et al.*, 1964b; Lloyd and Kabat, 1964; Lloyd *et al.*, 1966). Monosaccharides and their methyl or ethyl glycosides have been described earlier (Beychok and Kabat, 1965).

An insoluble derivative of purified hog gastric mucin with A + H blood-group activities (Kaplan and Kabat, 1966) was prepared by copolymerization with the *N*-carboxyanhydride of L-leucine (Tsuyuki *et al.*, 1956). This material is hereafter referred to as polyleucyl hog gastric mucin A + H (Pl-Hog A + H). The properties were those described earlier (Kaplan and Kabat, 1966).

Antisera against a crude extract of the albumin gland of *H. pomatia* were produced in rabbits. The animals were given three subcutaneous injections into the footpads of approximately 2.5 mg of snail protein emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The injections were given once a week and the animals were bled 6–8 days after the last injection.

Methods

Analytical Methods. Colorimetric methods for nitrogen, hexosamine, methylpentose (fucose), and hexose (galactose) 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 periodate uptake as previously described (Kabat, 1961).

Tentative identification and quantitation of carbohydrate in the purified snail hemagglutinin was performed by gas-liquid partition chromatography of the constituent sugars as their trimethylsilyl derivatives (Clamp *et al.*, 1967). The material was hydrolyzed in 1 M methanolic HCl at 80° for 24 hr with D-mannitol as an internal standard. Neutralization, re-N-acetylation, and trimethylsilylation of the released monosaccharides were performed as earlier described (Lloyd *et al.*, 1968) except that 6 hr was allowed for N-acetylation. Tri-

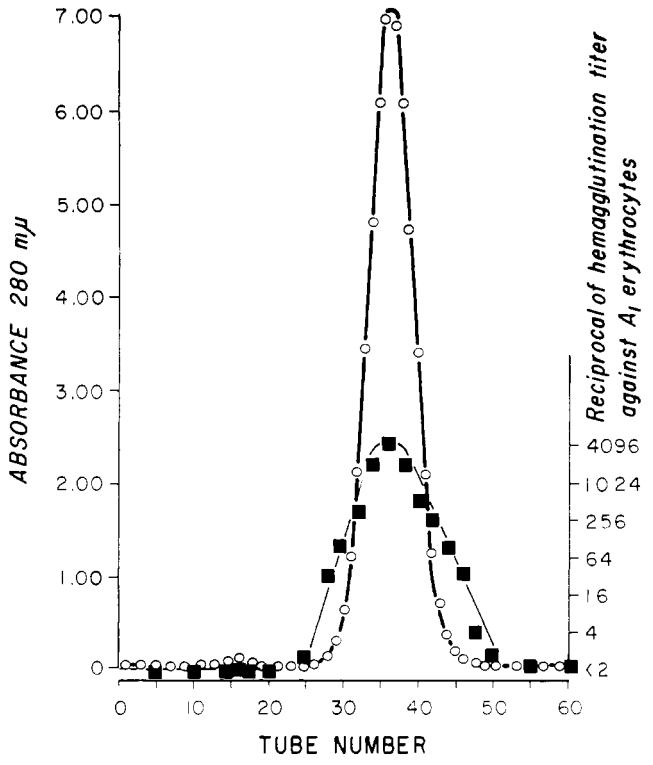

FIGURE 1: Gel filtration of purified snail hemagglutinin (fraction II) on Bio-Gel P-300 column. (○—○) Absorbance at 280 mμ; (■—■) reciprocal of hemagglutination titer against human A_1 erythrocytes.

methylsilyl derivatives of a standard mixture, previously subjected to acid hydrolysis under the same conditions as the sample and consisting of D-galactose, D-mannose, L-fucose, *N*-acetyl-D-glucosamine, and D-mannitol were also prepared. The compounds were separated on a SE-30, 80–100 S (10%) o a Diatoport S metal column (180 × 0.3 cm) using a F & M 810 gas chromatograph equipped with a flame ionization detector. The carrier gas flow was 40 cc/min. The temperature of the column was raised 1°/min from 138 to 220°. Areas of the peaks were calculated using a Disc Integrator and the ratio of each to the area of the internal standard was determined. By comparison with the standard sugar mixture, the amount of each sugar present was calculated.

Amino Acid Analysis.[1] Salt-free samples were hydrolyzed in constant-boiling HCl at 111° for 24, 48, and 72 hr over nitrogen in sealed Pyrex tubes. Quantitative analyses were performed by the technique of Spackman *et al.* (1958) using a Technicon amino acid analyzer. Methionine and half-cystine values were determined on samples hydrolyzed for 20 hr after prior oxidation with performic acid according to the method of Moore (1963). Tryptophan content was estimated from the tyrosine to tryptophan ratio determined by the method of Goodwin and Morton (1946).

Separation Procedures. Gel filtration employing Bio-Gel P-10 and P-300 columns was performed at 4° according to directions supplied by Bio-Rad Laboratories, Richmond, Calif. Ultracentrifugation was performed in a Spinco Model L centrifuge. Solutions were concentrated by vacuum dialysis using collodion bags (Schleicher & Schuell Co., Keene, N. H.).

Physicochemical Analysis. A Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and automatic temperature control was used. Sedimentation velocity experiments[2] were done at 50,740 rpm in double-sector cells with sapphire windows. The sedimentation coefficient at zero concentration was determined by extrapolation of the $s_{20,w}$ values from four runs at different concentrations (1.30–0.13 mg of N/ml) plotted as sedimentation coefficient *vs.* concentration. The intrinsic viscosity was calculated from viscosity measurements at different concentrations according to Schachman (1957) using an Ostwald capillary viscometer. The partial specific volume was calculated from the amino acid composition (McMeekin *et al.*, 1949).

The molecular weight was calculated from the relation between molecular weight, sedimentation coefficient, intrinsic viscosity, and partial specific volume using a value of 2.16×10^6 for the β function (*cf.* Gottschalk, 1966).

Disc electrophoresis in polyacrylamide gels was performed as described by Davis (1964) with modifications by Reisfeld and Small (1966). An alkaline buffer system, pH 9.3 (Reisfeld and Small, 1966), and a 10% gel concentration were employed. Electrophoresis was performed at a constant current of 1.0 mA/tube until the buffer line passed through the spacer gel into the small pore gel. The current was then increased to 2.5 mA/tube and electrophoresis was continued for 75 min after the buffer line had reached the end of the gel. Proteins were stained for 1 hr with 1% Amido Black in 15% acetic acid. Destaining was performed electrophoretically in 15% acetic acid.

Immunological Methods. Hemagglutination and hemagglutination inhibition experiments were 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 the addition of about 3–5 μg of hemagglutinin N were carried out by a microprecipitin technique using the ninhydrin procedure for nitrogen determination (Schiffman *et al.*, 1964a). Double diffusion in agar was performed in 1% gel (special Noble Agar, Difco Laboratories, Detroit, Mich.) containing 0.9% NaCl in 0.001 M phosphate buffer (pH 7.3) according to the method of Ouchterlony (1948).

Immunoelectrophoresis was carried out as described by Grabar and Williams (1953) at 150 V for 100 min using 1.5% agar containing 0.05 M sodium barbital buffer (pH 8.3).

Experimental Section and Results

Purification of Snail Hemagglutinin by Specific Absorption and Elution from a Pl-Hog A + H Column. Pl-Hog A + H (405 mg) was mixed with an equal amount of washed Celite, packed into a small column, and washed extensively with 0.9% NaCl. A dilute saline extract of the albumin gland of *H. pomatia* clarified by centrifugation at 15,000 rpm for 2 hr was then run through the column continuously until it was saturated, *i.e.*, until the optical density at 280 mμ and the hem-

[1] We are grateful to Drs. S. Vratsanos and B. F. Erlanger for performing these analyses.

[2] We are grateful to Dr. H. and Mr. S. Rosencrantz for performing these analyses.

TABLE II: Fractions Obtained by Elution with GalNAc and KSCN of Pl-Hog A + H Column Saturated with Snail Hemagglutinin.

Fraction	Agent Used for Elution (M)	Yield		Hemagglutinating Titer *vs.* A_1 Erythrocytes[b]
		mg of N	%[a]	
I	GalNAc (0.005)	3.6	20.6	$^1/_{256}$
II	GalNAc (0.005)	11.1	63.4	$^1/_{256}$
III	GalNAc (0.005)	1.9	10.9	$^1/_{256}$
IV	GalNAc (0.005)	0.57	3.3	$^1/_{128}$
V	GalNAc (0.015)	0.23	1.3	$^1/_{256}$
VI	GalNAc (0.05)	<0.01		ND
VII	KSCN (2.0)	0.12	0.7	$<^1/_2$

[a] Per cent of total eluted nitrogen. [b] Hemagglutination of fractions I–V and VII were performed with dilutions of solutions containing 154, 130, 120, 81, 115, and 104 μg of N per ml, respectively; titers represent highest dilution giving detectable hemagglutination.

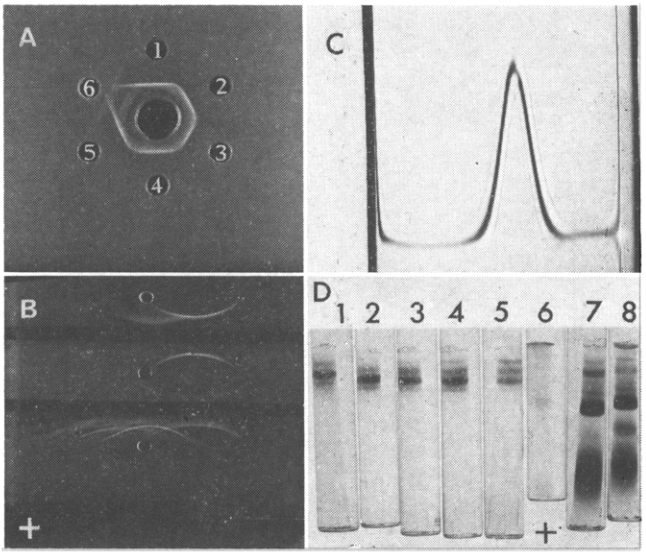

FIGURE 2: Immunodiffusion ultracentrifugal and electrophoretic patterns. (A) Immunodiffusion of fractions I–V and VII was obtained by specific elution with GalNAc or KSCN of Pl-hog A + H column saturated with snail hemagglutinin. Center well: rabbit antiserum against crude extract of albumin gland of *H. pomatia*. Peripheral wells: (1) fraction I, 221 μg of N/ml; (2) fraction II, 216 μg of N/ml; (3) fraction III, 200 μg of N/ml; (4) fraction IV, 202 μg of N/ml; (5) fraction V, 230 μg of N/ml; and (6) fraction VII, 208 μg of N/ml. (B) Immunoelectrophoretic pattern of specifically eluted snail hemagglutinin, fraction II (1.30 mg of N/ml, center well), and of crude extract of albumin gland (upper and lower wells); hog gastric mucin A + H (upper trough) and rabbit antiserum against crude extract of albumin gland (lower trough) were added to develop reactions. (C) Schlieren pattern of purified snail hemagglutinin (fraction II, 1.30 mg of N/ml in 0.9% NaCl). The photomicrograph was taken after 144 min at 50,740 rpm. (D) Disc electrophoretic pattern of purified snail hemagglutinin and of crude extract of albumin gland. (1) Fraction I, 8 μg of N; (2) fraction II, 7 μg of N; (3) fraction III, 7 μg of N; (4) fraction IV, 8 μg of N; (5) fraction V, 7 μg of N; (6) fraction VII, 5 μg of N; (7) partially purified A-active fraction of crude extract of albumin gland obtained by passing directly through Bio-Gel P-300 column, approximately 25 μg of N; (8) crude extract of albumin gland, approximately 25 μg of N.

agglutination titer of the eluate against human A_1 cells equaled that of the solution added. Complete saturation was reached with 410 ml of snail extract containing 60.6 μg of hemagglutinin N/ml. The column was then washed with 0.9% NaCl until the optical density at 280 mμ of the eluate was below 0.050. Hemagglutinin N (20.5 mg) was bound to the column as determined by the difference between the amount of snail hemagglutinin precipitated by antigen in the added extract and in the effluent and washings. The specific binding capacity of the Pl-hog A + H used is thus 32% on a weight basis, assuming that the hemagglutinin contains 16% nitrogen.

Specific elution of the hemagglutinin was effected by addition of GalNAc in 0.9% NaCl. Based on previous experiments a concentration of 0.005 M was chosen as the lowest concentration to elute most of the hemagglutinin within a reasonable time. As noted above the crude extract contains two components reacting with hog A + H. The second component, which does not agglutinate A_1 cells, seems to bind less firmly to the Pl-hog A + H column since it could be washed off by 0.9% NaCl.

As seen from Table II, 17.5 mg of nitrogen or 85.5% of the amount absorbed could be specifically eluted from the column; 98% of this was eluted by the 0.005 M GalNAc in one peak with a very extended tailing end (fraction I is the peak and fractions II to IV consecutive fractions of this tail). Elution with 0.015 M GalNAc (fraction V) gave a very small peak representing 1.3% of the specifically eluted nitrogen. Very little nitrogen was eluted with 0.05 M GalNAc and only 0.7% with 2.0 M KSCN (Avrameas and Ternynck, 1967; Dandliker *et al.*, 1967).

Each fraction was concentrated by ultrafiltration against several changes of 0.9% NaCl to remove most of the GalNAc. The last traces of GalNAc were subsequently removed by gel filtration on a Bio-Gel P-10 column (65 × 1.9 cm). None or only trace amounts of periodate-positive material could be detected. The protein peak was obtained immediately after the exclusion volume. After concentration it was rerun on a Bio-Gel P-300 column (61 × 1.9 cm) and the fractions were analyzed for absorption at 280 mμ and for hemagglutinating activity against human A_1 erythrocytes. All fractions except the KSCN eluate were treated in this way. Figure 1 shows the elution pattern obtained with fraction II. One symmetrical peak was found in the included volume. The hemagglutination titers for A_1 erythrocytes paralleled the protein concentration indicating that the same material is measured with both methods. The very small peak found in the excluded volume was devoid of hemagglutinating activity and was therefore not studied further. Identical elution patterns were found with fractions I, III, IV, and V.

All fractions except VII agglutinated human A_1 erythrocytes to the same titer when compared at approximately the same nitrogen concentration (Table II). Fraction VII, on the other hand, was inactive. None of the fractions agglutinated human B or O erythrocytes.

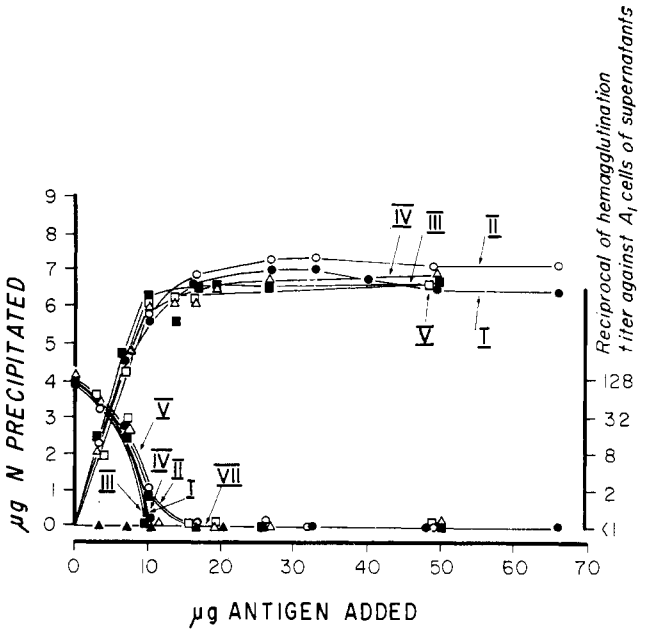

FIGURE 3: Precipitation by purified human blood-group A substance (MSM) of fractions I to V and VII obtained by specific elution with GalNAc or KSCN of Pl-hog A + H column saturated with snail hemagglutinin. (●—●) Fraction I, 6.27 μg of N/tube; (○—○) fraction II, 6.47 μg of N/tube; (■—■) fraction III, 5.93 μg of N/tube; (△—△) fraction IV, 6.06 μg of N/tube; (□—□) fraction V, 6.30 μg of N/tube; and (▲—▲) fraction VII, 4.25 μg of N/tube. The total volume was 200 μl.

TABLE III: Amino Acid Composition of Purified Snail Hemagglutinin (Fraction II).

Amino Acid	Residues/100,000 g			
	24 hr	48 hr	72 hr	Av[a]
Asp	89.9	90.5	93.8	91.4
Thr	46.0	44.6	44.2	47
Ser	79.3	73.8	64.9	87
Glu	60.9	61.6	60.3	60.9
Pro	50	52	54	52
Gly	37.6	37.4	36.5	37.2
Ala	43.3	44.5	42.2	43.3
Val	59.2	56.4	60.5	58.7
Cys[b]				23.3
Met[b]				12
Ile	46.3	47.4	49.8	47.8
Leu	48.4	47.0	50.1	48.5
Tyr	35.7	(27.5)	38.3	37.0
Phe	16.6	15.6	18.4	16.9
Lys	39.9	42.7	43.0	41.9
His	10.1	11.3	10.8	10.7
Arg	47.2	(44.3)	47.4	47.3
Trp[c]				35.8

[a] Threonine and serine values were obtained by a linear extrapolation to zero hydrolysis time. The 48-hr values for tyrosine and arginine are not included in the average. [b] Cysteine and methionine values were obtained from oxidized samples hydrolyzed for 20 hr. [c] Tryptophan was determined spectrophotometrically according to Goodwin and Morton (1946).

The purity of the specifically eluted fractions was assayed by immunological and physicochemical methods. Figure 2A shows the pattern obtained in gel diffusion against a rabbit antiserum prepared against the crude extract. Fractions I to V give one precipitin line showing complete fusion between the fractions while fraction VII gives four precipitin lines none of which fused with the other fractions. In addition, fraction IV gave a very faint line not visible on the photograph. The same pattern was obtained with a second antiserum against the crude extract. Both antisera were able to detect at least 15 different components in the crude extract (*cf.* bottom immunoelectrophoretic pattern, Figure 2B). All fractions were tested at the same nitrogen concentration (approximately 200 μg of N/ml).

Additional evidence for the purity of fractions I, II, III, and V was obtained in immunoelectrophoresis against hog gastric mucin A + H on one side and against the antisera mentioned above on the other. Figure 2B shows the pattern obtained with fraction II. Only one precipitin arc was obtained with both reagents. These precipitin lines fused and the position of the arc corresponded to about the γ region in the immunoelectrophoresis of serum.

As can be seen the crude extract contains two components able to react with hog gastric mucin and at least 15 different components detectable with antiserum. The diffuse appearance of the precipitin arc between the purified fraction and antiserum is due to the high concentration of fraction II used (1.30 mg of N/ml). Fractions I, III, and V gave identical results while fraction IV gave an additional very weak line with antiserum in the β region. Fraction VII, on the other hand, did not react at all with hog gastric mucin and showed four lines in the α–β region with antiserum.

In the analytical ultracentrifuge, fraction II sedimented as a single symmetrical peak with an $s^0_{20,w}$ of 5.28 S in 0.9% NaCl. No impurities could be detected at nitrogen concentrations up to 1.30 mg/ml (Figure 2C). The partial specific volume was 0.74 as calculated from the amino acid composition (Table III). The intrinsic viscosity of the fraction was 0.0675 dl/g. Since this value is lower than 0.1 dl/g, it is legitimate to use a value of 2.16×10^6 for the β function for calculating the molecular weight (*cf.* Gottschalk, 1966), which was found to be 1.0×10^5. Owing to shortage of material, none of the other fractions were investigated in the analytical ultracentrifuge.

Disc electrophoresis in polyacrylamide gels demonstrated that each fraction consisted of three or four components (Figure 2D). The same bands seemed to be present in all five fractions but in different concentrations. The main component seems to decrease in concentration from fractions I to V and only represents approximately one third of the material in fraction V as revealed by densitometric measurements. The other components show similar systematic changes from fractions I to V. The basis for this banding is not known. Fraction VII showed three bands, none of these corresponded to those found in the first five fractions.

Figure 3 shows quantitative precipitin curves obtained with fractions I to V and VII when tested against human blood-

TABLE IV: Inhibition of Hemagglutination between Snail Hemagglutinin and Human A_1 Erythrocytes with Blood-Group Substances, Monosaccharides, or Oligosaccharides.

Inhibitor	ABO Type	μg/ml Needed for Inhibition[a]
	Part a	
Cyst-MSM	A_1	5
Cyst-9	A_1	3.5
Cyst-14	A_2	34
Cyst-Beach	B	945
Cyst-19	B	56
Cyst-JS	O	142
Cyst-Tighe	O	77
Cyst-N-1-1	Le^a	498
Cyst-OG-3	—	48
Cyst-Beach P-1	—	375
Hog gastric mucin	A + H	8
	Part b	
Me-α-D-GalNAc		3.8
Et-β-D-GalNAc		>8.4[b]
D-GalNAc		6.8
Me-α-D-GNAc		21.6
Me-β-D-GNAc		>21.3[b]
Et-β-D-GNAc		99.0
D-GNAc		45.8
A_5II		>0.8[b]
AR_L 0.52		>1.4[b]

[a] 2–4 hemagglutinating units of snail hemagglutinin (fraction II) were used. [b] Highest concentration tested.

group A substance (MSM). The same amount of total nitrogen is precipitated by antigen at equivalence for the first five fractions while fraction VII does not give any detectable precipitation. Since the nitrogen content of blood-group A substance (MSM) is known, the amount of precipitable nitrogen in the fractions could be calculated; 93–101% of the nitrogen in fractions I to V was precipitable. These differences are within experimental error. Identity between the precipitating and hemagglutinating activities was demonstrated since supernatants showed a progressive decrease in titer with those in the equivalence zone giving no agglutination; the titer of the antigen-free control was $^1/_{128}$–$^1/_{256}$ (Figure 3).

Chemical Analysis of the Main Hemagglutinin Fraction (Fraction II). The amino acid composition of 24-, 48-, and 72-hr hydrolysates of fraction II was calculated on the basis of residues/100,000 g and the results are given in Table III, together with the average number of residues per mole (for calculations, see legend to Table III). It should be noted that the molecule contains cysteine and high concentrations of aspartic acid, glutamic acid, serine, valine, and arginine. No determination of amide content was performed.

Quantitative gas chromatography gave peaks corresponding to galactose (4.0%) and mannose (3.3%). A minor peak with a retention time equal to GalNAc and GNAc (0.4%) was also found. Colorimetrically calculated as galactose, 6.0% of the material reacted with the orcinol reagent. Colorimetric tests for methylpentose and sialic acids were negative.

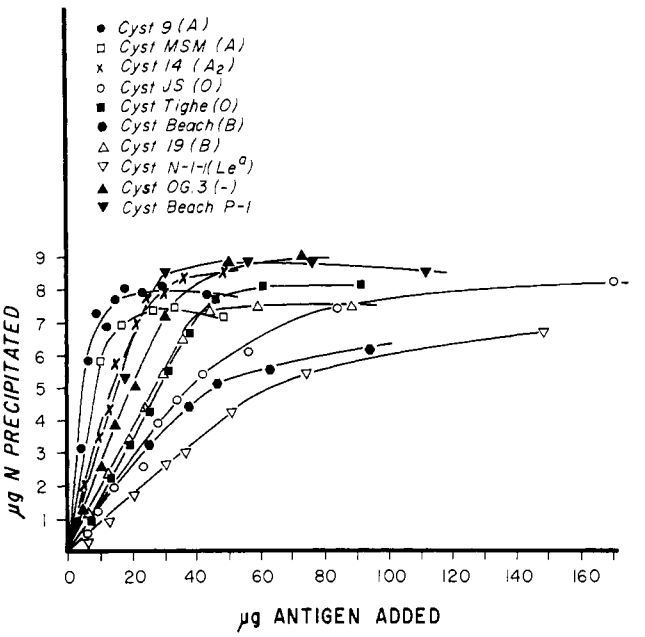

FIGURE 4: Precipitation of purified human blood-group substances from ovarian cysts by purified snail hemagglutinin. Fraction II (6.47 μg of N) was added to each tube; the total volume was 200 μl.

Immunologic Specificity of the Purified Snail Hemagglutinin (Fraction II). The reactivity of fraction II against human blood-group substances with A, B, H, or Le^a specificity is given in Figure 4. All cyst materials react with the snail hemagglutinin. However, the amount of each blood-group substance needed to precipitate a given amount of hemagglutinin varies. Thus, A_1 cysts (MSM and 9) were more active than the A_2 cyst (14) which was significantly more active than the B (Beach and 19), H (JS, Tighe), and Le^a (N-1) cysts. There are five points of interest. (1) Blood-group substances other than A precipitate with the hemagglutinin indicating that there is at least one other determinant in these cysts not identical with the A determinant. (2) B and O cysts with high and low reactivity can be found. (3) Only a fraction of the material in Beach (B), N-1 (Le^a), and Tighe (O) have the unidentified determinant in a form capable of reacting. This was shown by investigating the supernatants from the blood-group substance hemagglutinin precipitates in the antibody excess region; approximately 90% of the fucose and 85% of the hexosamine in cyst N-1 (Le^a) was found in such supernatants indicating that the material is a mixture and that only a small proportion precipitates with the snail hemagglutinin. Whether all B, O, and Le^a blood-group substances show similar heterogeneity is not known. (4) Treatment of B blood-group substance (Beach) with weak mineral acid to give the P-1 fraction (see Materials), which essentially removes fucose and oligosaccharides significantly increased its reactivity. The Beach P-1 has more than twice the activity of the original material on a weight basis. (5) An A, B, H, and Le^a negative cyst (OG) which had a fucose content of 2.4% reacted very strongly with the snail hemagglutinin.

By inhibition of the hemagglutination between fraction II

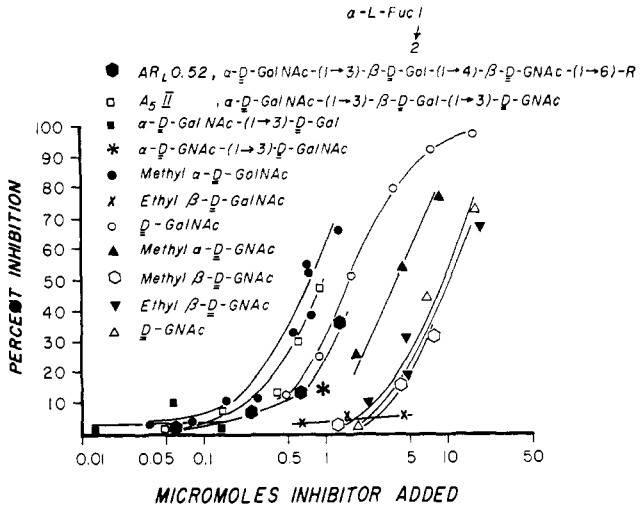

FIGURE 5: Inhibition by monosaccharides, methyl glycosides, and oligosaccharides of precipitation of human blood-group A substance with purified snail hemagglutinin. Fraction II (5.21 μg of N) plus A substance (12.8 μg) (MSM); total volume, 250 μl. The following compounds were also tested and gave no inhibition up to the amount indicated: β-D-Gal-(1→3)-D-GalNAc, 0.59 μmole; β-D-Gal-(1→3)-D-GNAc, 1.95 μmoles; β-D-Gal-(1→4)-D-GNAc, 2.09 μmoles; β-D-Gal-(1→6)-D-GNAc, 1.31 μmoles; β-L-Fuc-(1→3)-D-GNAc, 1.79 μmoles; Me-α-D-ManNAc, 5.32 μmoles; Me-β-D-ManNAc, 4.08 μmoles; D-ManNAc, 11.3 μmoles; Me-α-D-Gal, 10.9 μmoles; Me-β-D-Gal, 10.7 μmoles; D-Gal, 15.8 μmoles; Me-α-D-Glc, 8.5 μmoles; Me-β-D-Glc, 17.9 μmoles; D-Glc, 18.4 μmoles; Me-α-D-Man, 39 μmoles; D-Man, 22.3 μmoles; D-$GalNH_2$, 9.73 μmoles; D-GNH_2, 18.8 μmoles; and L-Fuc, 15.8 μmoles.

and human A_1 erythrocytes the same relative relationship in activity of the different cysts could be demonstrated (Table IVa) as was found by precipitation.

Figure 5 illustrates the ability of different monosaccharides, of their methyl α- and β-glycosides, and of some oligosaccharides to inhibit the precipitation between purified snail hemagglutinin (fraction II) and blood-group A substance (MSM). The best inhibitor is Me-α-D-GalNAc, which is approximately twice as good as D-GalNAc and about four and ten times better than Me-α-D-GNAc and D-GNAc, respectively. The ethyl β-D-glycoside of GalNAc did not inhibit even at very high concentrations. This is in contrast to the β-glycosides of D-GNAc which inhibited to the same extent as the unsubstituted sugar. No inhibition was obtained with other monosaccharides or their methyl glycosides including Me-α-D-Gal, Me-α-D-ManNAc, D-galactosamine, or D-glucosamine at concentrations higher than were needed to give 50% inhibition with D-GNAc. Among the disaccharides α-D-GNAc-(1→3)-D-GalNAc seemed to inhibit to the same extent as Me-α-D-GNAc while all other disaccharides tested were negative. Unfortunately the A-active disaccharide α-D-GalNAc-(1→3)-D-Gal was not available in quantities high enough to give an inhibition curve. It is, however, evident that this disaccharide is not a significantly better inhibitor than Me-α-D-GalNAc. The A-active tri- and pentasaccharides, A_5II and AR_L 0.52, inhibited precipitation at somewhat higher concentrations than Me-α-D-GalNAc when compared on a molar basis.

Inhibition of hemagglutination between purified snail hemagglutinin (fraction II) and human A_1 erythrocytes with the sugars used for inhibition of precipitation gave roughly the same inhibition pattern (Table IVb).

To establish that the terminal nonreducing GalNAc of the A determinant in human blood-group A substance was responsible for at least part of the reactivity in precipitation with snail hemagglutinin; the A-active ovarian cyst (MSS) was precipitated with the hemagglutinin, before and after treatment with a purified blood-group A N-deacetylating enzyme from *Clostridium tertium* (Marcus *et al.*, 1964) as well as with an enzyme-treated and subsequently re-N-acetylated sample. It is evident from Figure 6a that enzymatic N-deacetylation significantly increases the amount of blood-group substance needed for precipitation of a given amount of hemagglutinin. Re-N-acetylation restores the reactivity to the same level as that of untreated A substance.

The inhibition experiments raised the question of whether polysaccharides containing multiple terminal nonreducing D-GNAc might also precipitate with the snail hemagglutinin. This possibility was investigated using teichoic acids isolated from different strains of *S. aureus* and *albus*. The results are given in Figure 6b, teichoic acids from strains 3528, Copenhagen, and NYH-6, all containing α-linked D-GNAc, precipitated with the hemagglutinin while strain Al containing only β-linked D-GNAc did not react. The teichoic acid from *S. albus*, strain Prengel, which did not contain either α- or β-linked D-GNAc, was also negative. The amount of α-GNAc-polymer in strains 3528, NYH-6, and Copenhagen were previously found to be approximately 100, 50, and 15%, respectively (for references, see Materials). Interestingly the degree of reactivity of these teichoic acids with the hemagglutinin closely corresponds to the percentage of α-linked D-GNAc-polymer. Thus teichoic acids from strains NYH-6 and Copenhagen were one-half and one-sixth as active as the teichoic acid from the 3528 strain, respectively.

To investigate the homogeneity of the combining site of the hemagglutinin, inhibition of precipitation with Me-α-D-GalNAc, D-GalNAc, Me-α-D-GNAc, and D-GNAc was performed on fractions I, II, and IV using the cyst A substance MSM as antigen. Figure 7 gives the result of this experiment. For all three hemagglutinin fractions the relative degree of inhibition for the four sugars to one another was the same, within experimental error.

Discussion

The results obtained show that specific absorption of the snail hemagglutinin on columns of insoluble polyleucyl A + H substance and elution with *N*-acetyl-D-galactosamine is an excellent method for preparing the hemagglutinin in a highly purified state. It was free of contaminants when assayed at high concentrations both by immunoelectrophoresis and by double diffusion in agar against two different antisera to the crude extract. It was completely precipitated by human blood-group A substance, sedimented as a single symmetrical peak in the ultracentrifuge, and homogeneous on gel filtration.

The hemagglutinin, however, gave several bands on polyacrylamide gel electrophoresis at alkaline pH. The bands had similar mobilities and their relative concentrations appeared to change systematically from fractions I to V. Since all fractions, including V, were completely precipitated by A substance, it appears likely that all bands represent the hemag-

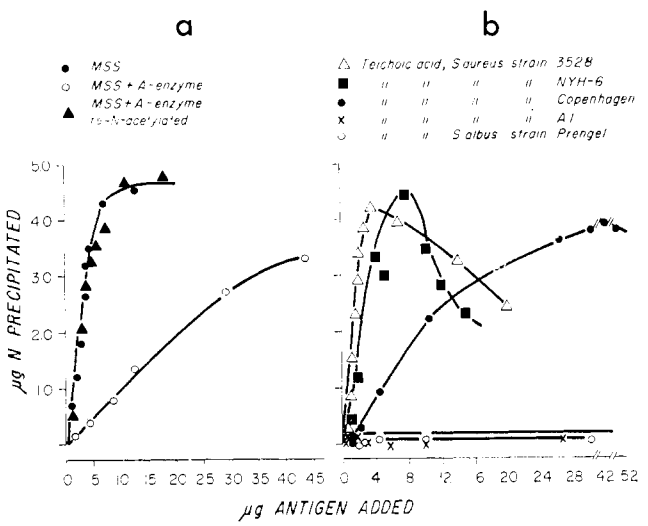

FIGURE 6: Precipitation studies. (a) Precipitation of purified snail hemagglutinin, 3.83 μg of N of fraction II, by human blood-group A substance prior to and after treatment with blood-group A N-deacetylating enzyme and of enzyme-treated and re-N-acetylated A substance; total volume, 200 μl. (b) Precipitation of teichoic acids from different strains of *S. aureus* and *S. albus* with purified snail hemagglutinin; 3.83 μg of N, fraction II; total volume, 200 μl.

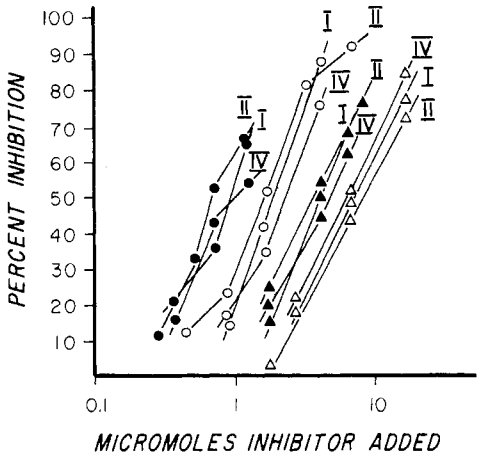

FIGURE 7: Relative effectiveness of four inhibitors in inhibiting precipitation of three different fractions of purified snail hemagglutinin (I, II, and IV) by human blood-group A substance (MSM); 13.6 μg of MSM plus 4.85 μg of N, fraction I; 12.8 μg of MSM plus 5.21 μg of N, fraction II; 12.8 μg of MSM plus 4.68 μg of N, fraction IV; total volume 200 μl. (●—●) Me-α-D-GalNAc, (○—○) D-GalNAc, (▲—▲) Me-α-D-GNAc, and (△—△) D-GNAc. The lines are drawn through each experimental point rather than as a straight line.

glutinin. The banding pattern could be the result of small differences in the chemical composition due to variation among individual molecules—pooled glands were used—or could be due to secondary changes such as partial deamidation brought about during handling. It is also possible that the hemagglutinin exists in multiple molecular forms.

The high yield (85.5%) of hemagglutinin, the absence of other contaminating proteins, and the finding that 93–101% was precipitable by blood-group A substance indicates the utility of the method.

The purified hemagglutinin had a molecular weight of about 100,000 as determined by sedimentation velocity and viscosity measurements. The amino acid composition (Table III) indicated that there are approximately 12 residues of methionine and 24 residues of half-cystine per mole of protein, the latter allowing for 12 disulfide bonds. Galactose, mannose, and traces of *N*-acetyl-D-galactosamine and/or *N*-acetyl-D-glucosamine were tenatively identified as their trimethylsilyl-ethers by gas–liquid partition chromatography. Recalculated as number of residues per mole of protein, 22.2, 18.5, and 1.8 residues were found for these monosaccharides, respectively. The traces of *N*-acetylhexosamine might be impurities (*e.g.*, *N*-acetyl-D-galactosamine from the elution step, not completely removed by ultrafiltration and gel filtration). It should be stressed that amino acids and monosaccharides found after acid hydrolysis of the hemagglutinin were only identified by their relative retention times compared with known standards and the identification should be regarded as tentative.

Inhibition of precipitation between snail hemagglutinin and human blood-group A substance showed that Me-α-D-GalNAc is the best inhibitor of those tested. Since the A-active di-, tri-, and pentasaccharides did not inhibit at lower molar concentrations, this suggests that the combining region of the hemagglutinin may be no larger than the size of a monosaccharide plus the α linkage. The possibility, however, that none of the compounds tested represents the true complementary structure should be kept in mind.

The hemagglutinin is not absolutely specific for α-linked D-GalNAc since inhibition was also obtained by the methyl α- and β-glycosides of D-GNAc at four and ten times higher molar concentrations, respectively. The importance of the stereochemical arrangement of the acetamido group on carbon 2 of the pyranose ring is evident since D-ManNAc or Me-α-D-ManNAc was inactive as inhibitors indicating that the acetamido group must be oriented equatorially to interact with the combining site. The importance of the acetyl group is also seen in that neither D-galactosamine nor D-glucosamine inhibited at comparable concentrations. Axial orientation of the hydroxyl group on carbon 4 is preferred since D-GalNAc is a better inhibitor than D-GNAc. However, there is not the same strict discrimination between axial or equatorial orientation of this hydroxyl as for the acetamido group on carbon 2. This is perhaps not surprising considering the differences between the two groups. Axial orientation of the hydroxyl on carbon 1 is preferred since the methyl α-D-glycoside of GalNAc is a much better inhibitor than the corresponding methyl β-D-glycoside. Indeed, no inhibition could be demonstrated with Et-β-D-GalNAc even at concentrations at which D-GNAc alone gave significant inhibition. This is of interest since Me- or Et-β-D-GNAc inhibited at these concentrations and were as active as D-GNAc. The reason for this difference is not clear. No information regarding the preferred orientation of substituents on carbons 3 and 5 can be obtained from these data. D-GalNAc and D-GNAc probably must be at the nonreducing end in oligosaccharides since the disaccharides β-D-Gal-(1→3)-D-GNAc, β-D-Gal-(1→4)-D-GNAc, β-D-Gal-(1→6)-D-GNAc, β-D-Gal-(1→3)-D-GalNAc, or β-L-Fuc-(1→3)-D-GNAc were inactive.

From the inhibition data it would appear that macromolecules containing α-linked D-GalNAc residues and possibly also those containing either α- or β-linked D-GNAc, but not β-

linked D-GalNAc, as the terminal nonreducing sugars would precipitate with the snail hemagglutinin. Precipitin analyses of human blood-group A substance from ovarian cyst fluids, before and after treatment with a purified blood-group A N-deacetylating enzyme from *Cl. tertium* (Marcus *et al.*, 1964) and of enzyme-treated and subsequently re-N-acetylated A substance (Marcus *et al.*, 1964), demonstrated that the snail hemagglutinin requires the *N*-acetyl group of D-GalNAc on the A substance for optimal reactivity (Figure 6a). Since the reactivity of the snail hemagglutinin parallels that of human anti-A when tested against the same preparation (MSS) (Marcus *et al.*, 1964) and since DNP-galactosamine was isolated on hydrolysis of enzyme-treated blood-group A substance after reaction with dinitrofluorobenzene (Marcus *et al.*, 1964), it can be concluded that α-linked D-GalNAc is responsible for the main reactivity of the hemagglutinin with human A substance.

As can be seen from Figure 6a, the snail hemagglutinin gave some precipitation with the enzyme-treated preparation. This reactivity probably involves the other determinant which reacts in blood-group substances with B, H, and Le^a specificities and with the precursor substance (Cyst OG) (Figure 4). Whether this determinant is the same in all blood-group substances including the A substance, is at present not known. The finding that low fucose content as in OG substance or as in the B-active cyst treated with weak acid to give the P-1 fraction (Allen and Kabat, 1959) correlates with increased reactivity with hemagglutinin is especially significant since it might indicate that a residue either sterically obstructed by or substituted by fucose is responsible for this reactivity. It is also of considerable interest that all non-A cysts tested consisted of two populations of molecules, one reactive and one nonreactive with the hemagglutinin. It is probable that the reactive population contains incompletely biosynthesized material in which underlying structures remain exposed and are responsible for the reactivity and possibly that some degradation occurred while in the cyst cavity or in subsequent handling.

The question of whether macromolecules with α- and β-linked D-GNAc can react with the hemagglutinin was resolved by the studies with teichoic acids. Precipitin analyses with purified hemagglutinin and teichoic acids from different strains of *S. aureus* demonstrated conclusively that only those teichoic acids containing D-GNAc linked α to the ribitol phosphate backbone are precipitated (Figure 6b). The teichoic acids from strain Al, containing only β-D-GNAc, did not precipitate, while the strains containing mixtures of α and β-linked D-GNAc reacted proportionally to their content of α-D-GNAc teichoic acid. It is surprising that teichoic acids with β-linked D-GNAc did not precipitate with the hemagglutinin although precipitation with A substance was inhibited with high concentrations of Me-β-D-GNAc. Perhaps the small methyl group on the Me-β-D-GNAc does not prevent the sugar from entering into the combining site but when the D-GNAc is attached to a ribitol backbone some steric hindrance occurs with the β but not with the α anomer. This reaction provides a precise estimate of the proportion of α- to β-linked D-GNAc teichoic acids and the snail hemagglutinin may prove quite useful in this respect with other macromolecules. Indeed, it has proven better than optical rotatory dispersion (Kabat *et al.*, 1969) for quantitative estimation of small proportions of α-linked D-GNAc.

The finding that α-linked D-GNAc, which is a considerably poorer inhibitor than α-linked D-GalNAc, nevertheless can precipitate when present in multivalent form on a macromolecule, shows that optimum binding is not a necessary requirement for precipitation. It would be important to establish how weakly a given sugar could bind to a site and yet be able to precipitate when present in polymeric form without steric or other factors restricting access to the site.

The combining sites of the hemagglutinin appear to be homogeneous as revealed by the inhibition of precipitation assays using four different inhibitors with three fractions of the hemagglutinin eluted with 0.005 M D-GalNAc but coming from different regions of the elongated elution peak. As shown in Figure 7, all four inhibitors gave the same degree of inhibition relative to Me-α-D-GalNAc with each fraction. If the combining site had been heterogeneous, one would have expected that the less readily eluted fractions would show different patterns of inhibition with these sugars as compared with the more easily eluted ones. This finding is of considerable interest since fractionated antibody populations previously investigated by this method showed differences in relative inhibiting power for a given set of inhibitors when different fractions were assayed (*cf.* Kabat, 1968) and validates hapten inhibition for establishing both homogeneity and heterogeneity of specific sites.

The large number of proteins which react more or less specifically with carbohydrate determinants should, when more extensively investigated, provide a series of reagents which can recognize specific linkages and individual sugars in polysaccharides comparable with what has been done in studying cross-reactions with antipneumococcal antibodies (*cf.* Heidelberger, 1965).

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