STUDIES ON HUMAN ANTIBODIES

IV. Purification and Properties of Anti-A and Anti-B Obtained by Absorption and Elution from Insoluble Blood Group Substances*

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Antibodies formed in response to injection of single antigens may be extraordinarily heterogeneous, often consisting of molecules belonging to each of the three major classes of serum immunoglobulins, γG, γM, and γA[1] (1–3). McDuffie, et al. (7), studying the properties of human blood group isoantibodies, showed that rabbits immunized with specific precipitates of A substance and human anti-A developed antibodies to three serum proteins; two of these were identified as antibody to γG- and γM-immunoglobulins. The third protein remained unidentified until it was characterized by Kunkel and Rockey (8) as γA. Serum from individual human donors may contain anti-A or anti-B of all three immunoglobulin classes (8–10).

It would be of great interest to determine the characteristics of the combining sites of the various anti-A immunoglobulins formed in a single individual. The present study reports a new method for purifying anti-A or anti-B using specific insoluble adsorbants, separation of the purified anti-A antibodies into γG- and γM-fractions, and initial observations on the relative capacities of various oligosaccharides involved in the A antigenic determinant to inhibit the precipitation of γG– and γM–anti-A by blood group A substance.

Materials and Methods

Blood Group Substances.—Soluble blood group substances were purified from hog gastric mucin, (Wilson Laboratories, Chicago), and human ovarian cysts by previously described

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[1] The nomenclature used is that recommended by the World Health Organization (4). γD (IgD), recently described by Rowe and Fahey (5), has not yet been shown to exhibit antibody activity (6).

methods (11). Insoluble derivatives of these purified blood group substances were prepared by copolymerization with the *N*-carboxyanhydride of L-leucine as described by Tsuyuki, et al. (12).[2] The details of this procedure were as follows: Purified blood group substance was dissolved to a concentration of 4 mg/ml in $\frac{7}{100}$ M $NaHCO_3$ (pH 8.3). The solution in an Erlenmeyer flask was chilled to approximately 5°C and mixed continuously by magnetic stirring. To it was slowly added solid crystalline *N*-carboxy-L-leucine anhydride (Pilot Chemicals, Inc. Watertown, Massachusetts) in an amount equal to the weight of dissolved blood group substance. Stirring in the cold was continued for an additional 48 hr. The contents of the flask were then centrifuged for 1 hr at 17,000 RPM in a refrigerated Servall RC-2 centrifuge. The clear supernatant solution was decanted, the precipitate thoroughly dispersed in 25 volumes of $\frac{7}{100}$ M $NaHCO_3$ and again centrifuged. This step was repeated until the precipitate had been washed 4 times in $NaHCO_3$ and 4 times in distilled water. After the last wash the precipitate was lyophilized and stored at room temperature. The insoluble blood group materials so prepared are, hereafter, referred to as polyleucyl blood group substances (Pl-BGS) and as polyleucyl A (Pl-A) and polyleucyl B (Pl-B).

Inhibitors.—Haptenic derivatives of purified human ovarian cyst A substance were prepared by mild acid hydrolysis (13) and by alkaline cleavage in the presence of sodium borohydride (14). Two oligosaccharides, A_5II (13), a trisaccharide having the structure:

$$\alpha\text{-D-GalNAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal-}(1 \rightarrow 3)\text{-D-GNAc}$$

and AR_L 0.52 (previously called A_3) a pentasaccharide with the structure:

$$\begin{array}{c} \alpha\text{-L-Fuc } 1 \\ \downarrow \\ 2 \\ \alpha\text{-D-GalNAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal-}(1 \rightarrow 4)\text{-}\beta\text{-D-GNAc-R} \end{array}$$

representing the largest and most active fragment of the antigenic determinant of blood group A substance thus far isolated (15–17) were utilized along with *N*-acetyl-D-galactosamine (GalNAc) to inhibit the precipitation of the purified anti-A by A substance as described earlier (18, 19, 13).[3]

Antisera.—Serum R.G., generously provided by Dr. Richard E. Rosenfield of Mount Sinai Hospital, New York, was obtained from a multiparous blood group O female hyperimmunized by repeated blood group A pregnancies. Two separate bleedings from this donor were obtained, the first (R.G.I) dating from June, 1959, and the second (R.G.II) from February, 1962. Serum Ortho 63-2622 was the gift of Dr. Philip Levine and Mr. Glen Hill of Ortho Research Laboratories, Raritan, New Jersey. The donor of this serum was a blood group B individual immunized with hog A substance. These sera were preserved with phenol (0.25%) and merthiolate (1:10,000) and were stored at 4°C. Goat and rabbit antisera to whole human serum and to human γG-, γA-, and γM-immunoglobulins were purchased from Hyland Laboratories, Los Angeles, and from Lloyd Bros., Inc., Cincinnati. Rabbit antisera specific for human kappa (group I) and lambda (group II) polypeptide chains were the gift of Dr. Henry Kunkel of The Rockefeller University. Dr. John Fahey of the National Institutes of Health generously furnished rabbit antiserum to human γD and serum W.T., a serum containing known quantities of γD. Antisera specific for human complement components C'q and β_1C/β_1A were kindly supplied by Dr. Charles Christian and Dr. Konrad Hsu of the College of Physicians and Surgeons.

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[2] We are indebted to Dr. Lawrence Levine of Brandeis University who suggested the possible applicability of this method.

[3] GNAc, *N*-acetyl-D-glucosamine; Fuc, fucose; and Gal, galactose.

Carbohydrates.—D-Galactose and sucrose were purchased from Pfanstiehl Chemicals, Waukegan, Illinois, and *N*-acetyl-D-galactosamine from Nutritional Biochemicals Corp., Cleveland. Biogel P 10 and P 300 used for gel filtration were obtained from Bio-Rad Laboratories, Richmond, California.

Analytical Methods.—Standard analytical techniques were utilized and the following substances measured by the methods given: methylpentose, Dische and Shettles; hexosamine, Elson and Morgan; and reducing sugar, Park and Johnson (cf. 18). The procedure for nitrogen was a modification (14) of the method of Rosevear and Smith (20).

Separation Procedures.—Gel filtration employing Biogel P 10 and P 300 columns was carried out at room temperature according to the directions supplied by Bio-Rad Laboratories.

Density gradient ultracentrifugation was performed as described by Kunkel (21) utilizing a 4 ml linear (10 to 40%) sucrose gradient in a Spinco model L centrifuge with SW 39 rotor for 16 hr at 35,000 RPM.[4] Ten fractions of equal volume (*circa* 0.45 ml) were collected dropwise through a pin hole made in the bottom of the tube.

Immunochemical Methods.—Hemagglutination studies were performed with a Takatsy microtitrator (Cooke Engineering Company, Alexandria, Virginia) using 0.025 ml loops and 2% erythrocyte suspensions at room temperature.

Quantitative precipitin analyses (18) were performed by a microprecipitin technique using the ninhydrin procedure for nitrogen (14).

Agar diffusion studies were carried out at 37°C according to the method of Ouchterlony (22) in 1% gels (Ionagar No. 2, Consolidated Labs., Inc., Chicago) containing 0.5 M glycine (23) and 1:10,000 merthiolate.

Immunoelectrophoresis was performed as described by Grabar and Williams (24) (cf. 25).

Purification of Isohemagglutinins.—The principle of the method involves the specific absorption of anti-A or anti-B antibodies by Pl-A or Pl-B respectively, removal of nonspecific protein by washing, and elution of absorbed antibody by extraction with acid buffer or with sugar haptens at neutral pH. The specific procedure was as follows:

Absorption: To a given volume of serum containing anti-A was added 1 mg/ml of Pl-A. The serum was thoroughly mixed to suspend the insoluble material evenly, and incubated at 37°C for 1 hr with frequent mixing. The serum was then slowly rotated (10 to 16 RPM) for 1 wk in the refrigerator. The insoluble Pl-A was harvested by centrifugation and the supernatant serum tested for residual anti-A by hemagglutination and by quantitative precipitin tests. It was invariably found that more than 90% of the homologous antibody had been removed by the Pl-A. With R.G., a type O serum which contained both anti-A and anti-B, absorptions were performed in 2 different ways: in the first instance the serum was absorbed only with Pl-A; in the second experiment sequential absorptions with Pl-B followed by Pl-A were carried out.

After absorption, the Pl-BGS–antibody complex was washed repeatedly with cold saline until the washings were essentially devoid of material absorbing at 2800 A.

Elution: Elution of antibody was performed by suspending the packed, washed Pl-BGS–antibody precipitate in 2 ml, M/10 acetate buffer, pH 3.62 at 0°C (26), for 1 hr with frequent mixing. After centrifugation at 4°C, the supernatant was immediately transferred to a dialysis casing unknotted at the top and dialyzed against 500 ml cold PO_4-buffered saline (M/20, pH 7.3). Fresh, cold acetate buffer (pH 3.62) was reapplied to the packed sediment and the extraction repeated. The second eluate was added to the first in the dialysis bag, and the dialysis fluid changed. A third elution was performed and the Pl-BGS washed several times with small quantities of saline. The combined eluates and saline washes were dialyzed for 6 hr against 1

[4] We gratefully acknowledge the expert assistance of Dr. K. Aho in the performance of these studies.

liter of M/20 PO_4-buffered saline (pH 7.3) and overnight against M/1000, PO_4-buffered saline (pH 7.3). Since the antibody yield by acid elution did not exceed 10%, the procedure was changed as follows: washed, packed Pl-A that had been used for anti-A absorption was suspended in 2 to 3 ml of PO_4-buffered saline (M/1000, pH 7.2–7.3) containing 1 g of GalNAc, incubated for 1 hr at 37°C, and slowly rotated overnight in the refrigerator. After centrifugation, the supernatant eluate was removed and the Pl-A resuspended in additional GalNAc in buffered saline. This procedure was repeated twice and the resultant eluates pooled. The Pl-A was washed several times in cold, buffered saline and the washings added to the pooled eluates. The elution procedure for Pl-B was identical to that for Pl-A except that D-galactose was substituted for GalNAc. The pooled eluates and washings were concentrated by ultrafiltration in the cold under negative pressure through an 8 ml collodion membrane, porosity less than 5 mμ (Membranfiltergesellschaft Göttingen, Germany, obtained from Schleicher and Schuell, Keene, New Hampshire). Initially, the concentrated, sugar-containing eluates were freed of

TABLE I

Analytical Properties of Purified Blood Group Substances and of Their Insoluble Polyleucyl Derivatives

Material	Methylpentose		Hexosamine	
	%	% BGS	%	% BGS
Hog A	10.6	100	29.0	100
Pl-hog A (I)	5.8	55	16.3	56
Pl-hog A (II)	6.0	57	15.3	53
McDon A	21.1	100	30.9	100
Pl-McDon A	7.6	36	11.1	36
Beach B	19.3	100	21.3	100
Pl-Beach B	8.7	45	9.4	44

sugar by repeated cycles of dilution with buffered saline and ultrafiltration. More recently it was found by Dr. Marianne M. Dorner in this laboratory, that a single passage of such a concentrated eluate through a Biogel P-10 column (1.1 × 60 cm) equilibrated with buffered saline (M/1000, pH 7.2) served to separate completely hapten (i.e. sugar) from protein. Those chromatographic fractions absorbing at 2800 A were pooled and reconcentrated by ultrafiltration.

RESULTS

Preparation of and Absorption with Insoluble Blood Group Substances.—Table I summarizes the analytical properties of the original purified blood group substances and of their insoluble polyleucyl derivatives. Two separate batches of Pl-hog A (I and II) were prepared from the same starting materials. When the methylpentose and hexosamine contents of the various polyleucyl compounds are expressed as percentages of those found in the parent blood group substances, it is apparent that the insoluble derivatives contained from 36% (Pl-Beach B) to about 55% (Pl-hog A) of blood group material.

The capacities of the polyleucyl blood group substances to absorb specifically anti-A and anti-B from human sera is illustrated in Table II. Absorption of R.G.I, which contained both anti-A and anti-B, with Pl-hog A was found to remove 95% of the anti-A (22 of 23 μg Ab (antibody) N/ml) and about 75% of the anti-B (3.6 of 4.7 μg Ab N/ml). The absorbed serum agglutinated type B cells to a titer of 1:16 but, even undiluted, would no longer agglutinate type A_1 erythrocytes. Pl-McDon A absorbed from serum R.G.I more than 95% of the antibody N which McDon A was capable of precipitating (13.5 of 14.0 μg Ab N/ml). This represented only about ¾ of the total anti-A precipitable by hog mucin A. Pl-A absorption removed most of the anti-B from R.G.I as well.

TABLE II

Absorption of Anti-A and Anti-B by Insoluble Polyleucyl Blood Group Substances

Antiserum	Heamgglutination titer		Antibody N precipitated by		
	A cells	B cells	Hog A	McDon A	Beach B
			μg/ml	μg/ml	μg/ml
R.G.I					
Unabsorbed	128	32	23.0	14.0	4.7
Absorbed with Pl-hog A	0	16	1.0	0.7	1.1
Absorbed with Pl-McDon A	2	16	5.0	0.5	1.8
R.G.II					
Unabsorbed	128	32	18.4		4.7
Absorbed with Pl-Beach B	64	0	17.1		0.2
Absorbed with Pl-Beach B + Pl-hog A	0		0.5		
Ortho 63-2622					
Unabsorbed	256	0	13.3		
Absorbed with Pl-hog A	0		0.9		

Serum R.G.II, containing 20% less anti-A than R.G.I, was absorbed first with Pl-Beach B and then with Pl-hog A. The first absorption removed more than 95% of the antibody N precipitable by human B substance, (4.5 of 4.7 μg Ab N/ml) but less than 10% of the Ab N precipitable by hog A (1.3 μg of a total of 18.4 μg Ab N/ml). The rest of the anti-A was removed by a subsequent absorption with Pl-A. These findings indicate that in serum R.G., AB cross-reacting antibody makes up a large proportion of the anti-B and only a small proportion of the anti-A.

Purification and Characterization of Blood Group Antibodies.—Table III summarizes the absorption and elution steps used in isolating anti-A and anti-B, the yields obtained, and the agglutinating and precipitating properties of the purified antibodies. In the initial study suboptimal quantities (0.5 mg/ml

serum) of Pl-A were used for absorption, and elution was performed with pH 3.62 acetate buffer. An overall anti-A yield of only 7.3% was achieved. Subsequently, antibody recoveries were improved by absorbing with larger quantities of Pl-A and eluting with GalNAc at neutral pH.

Both the acid and GalNAc eluates prepared from Pl-A-R.G.I antibody complex agglutinated type B cells to a modest titer, 1:16; however, neither eluate was precipitated by human B substance.

In an attempt to isolate AB cross-reacting antibody from serum R.G.II, the latter was absorbed with Pl-B and elution carried out with galactose, the terminal nonreducing sugar of the B antigenic determinant (19, 27). The re-

TABLE III *Preparation and Properti*

Serum	Volume used	Total antibody N		Absorption				
					Residual antibody N			
		Anti-A	Anti-B	Absorbant	Anti-A		Anti-B	
	ml	*μg*	*μg*		*μg*	%	*μg*	%
R.G.I.	125	2875	563	Pl-hog A*	688	24	N.D.‡	
R.G.I.	45	1035	212	Pl-hog A	45	4	45.2	21
R.G.II	(*a*) 160	2950	768	Pl-Beach B	2735	93	40	5
	(*b*) 150	2565	38	Pl-hog A	75	3	N.D.	
Ortho 63-2622	225	2990		Pl-hog A	214	7		

* In this instance only 0.5 mg Pl-hog A/ml serum was used for absorption which a counts for the incomplete removal of anti-A.

‡ Not determined.

sultant eluate, although relatively poor in nitrogen, agglutinated type A and B erythrocytes to titers of 1:256 and was incompletely precipitated by both A and B substances. Since no effort was made to differentiate the anti-B from anti-A B, it is not possible to relate, quantitatively, the N content of this eluate to the anti-A or anti-B present in the original serum. Efforts to obtain additional antibody from the Pl-B substance by elution with galactinol were unsuccessful.

Fig. 1 A and 1 B demonstrate that Pl-A-GalNAc eluates from R.G.II and Ortho 63-2622 contained γG-, γM-, and γA-immunoglobulins. (All three immunoglobulins were also detected in the Pl-B-Gal eluate from serum R.G.II.) When the immunoelectrophoretic patterns were developed with polyvalent goat anti-human serum, some of the eluates were found to contain trace quantities of albumin; furthermore, the Ortho eluate gave an additional very faint band in the slow β-region close to, but separate from, the γM-line. The sub-

stance responsible for the "extra" precipitin band is unknown. This eluate was tested for γD (Fig. 1 C) and complement components C'q and β1C/β1A, but they were not found.

Fig. 1 D demonstrates the precipitation in agar gel of hog and human A blood group substances by the concentrated R.G.II anti-A eluate. A continuous arc of precipitation, without spurring, developed (wells 1 and 6). This indicates that although in the cold hog A precipitated 40% more AbN from serum R.G.II, than did McDon A, with respect to the antibodies present in this anti-A eluate, hog A and McDon A substances are antigenically indistinguishable within the sensitivity of the agar diffusion method. The precipitin lines formed by the

Anti-A and Anti-B Eluates

Elution			Eluate							
Eluant	Total N	N recovery	Ab N pptable by A subst.	Ab N pptable / total N	Recovery of Anti-A	Ab N pptable by B subst.	Ab N pptable / total N	Recovery of anti-B	Hemagglutination titer	
									A cells	B cells
	μg	%	μg	%	%	μg	%	%		
I 3.62	306	11	209.5	69	7	0	0	0	512	16
lNAc	351	34	254	72	25	0	0	0	256	16
lactose	143	?	38.4	?	?	39.3	?	?	256	256
NAc	1279	50	910	71	34	0	0	0	1280	0
lNAc	2128	71	1125	53	38				5120	

eluate and anti-γG, anti-γM, and anti-γA all penetrated the A-anti A band. The latter, in turn, spurred through the anti–γM- and anti–γA but not through the anti–γG-lines. These findings indicate that the R.G.II anti-A eluate contains molecules of each immunoglobulin class not precipitable by A substance in agar at 37°C. In contrast, quantitative precipitin studies performed at 0°C showed the eluate to be 75% precipitable by A substance. The failure of the A-anti A band to penetrate the eluate–anti-γG-precipitin line may reflect the reactivity of the unabsorbed anti–γG-serum with L chain determinants common to all three immunoglobulin classes (2).

Fractionation of Purified Blood Group Antibodies.—

Ortho eluate: An aliquot containing 1735 μg N, of the Ortho eluate was concentrated to 1.5 ml and 150 mg of crystalline sucrose added. The solution was layered at the liquid-gel interface of a 20 × 700 mm Biogel P-300 column, equilibrated at room temperature with M/3.3 buffered saline, pH 7.4. 1 ml

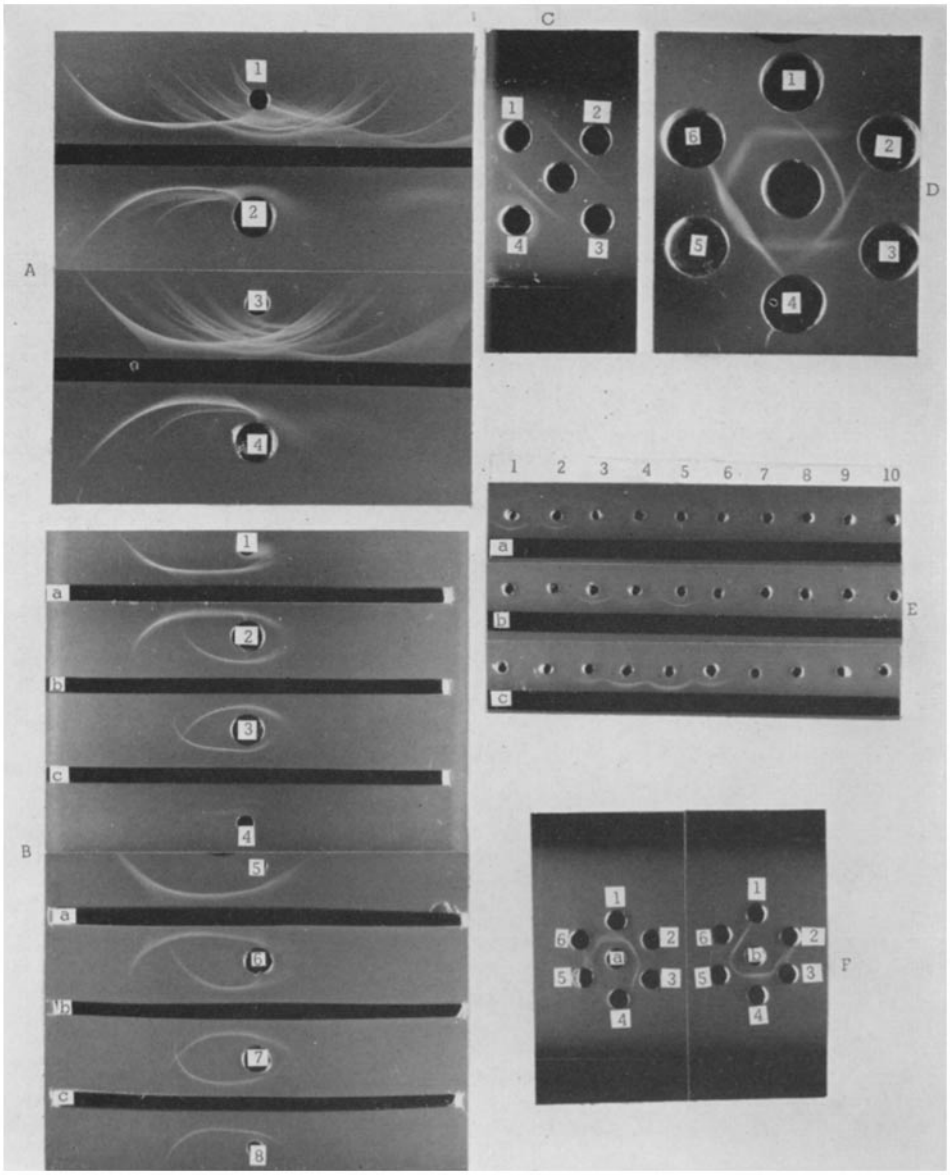


FIG. 1. A. Immunoelectrophoretic patterns of (1) Ortho 63-2622 serum, (2) Ortho-GalNAc eluate, (3) R.G.II serum, and (4) R.G.II-GalNAc eluate; goat anti-human serum was used in the troughs to develop reactions.

B. Immunoelectrophoretic patterns of (1, 4) Ortho 63-2622 serum; (2, 3) Ortho-GalNAc eluate; (5, 8) R.G.II serum; and (6, 7) R.G.II-GalNAc eluate; troughs (*a*) goat anti–γG-globulin, (*b*) goat anti–γA-globulin, and (*c*) goat anti–γM-globulin.

C. Center well: rabbit anti–γD-serum. Peripheral wells, (1) empty, (2) Ortho 63-2622 serum, (3) Ortho-GalNAc eluate, and (4) W.T. serum (γD-standard).

D. Center well: R.G.II-GalNAc eluate. Peripheral wells, (1) McDon A substance, (2) goat anti–γM-globulin, (3) goat anti–γA-globulin, (4) McDon A substance, (5) goat anti–γG-globulin, and (6) hog A substance.

E. Immunodiffusion study of ten fractions resulting from density gradient centrifugation of R.G.II-GalNAc eluate. Trough (*a*) goat anti–γM-globulin, (*b*) goat anti–γA-globulin, and (*c*) goat anti–γG-globulin.

F. Center well (*a*): rabbit anti–type K (group I) antiserum. Center well (*b*): rabbit anti–type L (group II) antiserum. Peripheral wells, (1) type K Bence-Jones protein, (2) R.G.II γG-fraction, (3) R.G.II γM-fraction, (4) type L Bence-Jones protein, (5) Ortho γM-fraction, and (6) Ortho γG-fraction.

fractions were collected at the rate of 18 per hour and their absorbance at 2800 A measured. The absorption curve showed two broad, incompletely separated peaks. The immunoglobulins present in selected fractions were characterized by means of agar diffusion employing γM-, γG-, and γA-specific antisera. Second peak fractions 68 to 137 containing γG and very small amounts of γA, were pooled, dialyzed against isotonic buffered saline, and concentrated by ultrafiltration to give the γG-fraction of the Ortho eluate. Fractions 33 to 55, which represented the ascending limb of the first protein peak, were found to be preponderantly γM; however, after these fractions had been pooled and concentrated, trace quantities of γG and γA were detected. Consequently, P-300 gel filtration was repeated under the conditions previously described. Only a single

TABLE IV

Properties of the Unfractionated Anti-A eluates and of Their Separated γG- and γM- Fractions

Eluate	Nitrogen		OD 2800 A	OD 2800 A / μg N/ml	Ab N pptable by A subst.	Ab N pptable / total N	Anti-A hemagglutination titer	μgN/ml / titer
	μg	μg/ml			μg	%		
Ortho unfractionated*	180	90.2	0.890	0.00987	91.4	51	320	0.28
γG-fraction	625	68.7	0.655	0.00953	326	52	320	0.21
γM-fraction	94	62.8	0.520	0.00829	40.7	43	160	0.39
R.G.II unfractionated	985	365	N.D.	N.D.	702	71	5120	0.07
γG-fraction	498	148.8	1.492	0.0103	343	69	5120	0.03
γM-fraction	112	34.1	0.440	0.129	99.5	89	80	0.43
Intermediate fraction	15	12.7	0.141	0.111	10.7	71	80	0.16

* This represents a dilution of a small portion of the Ortho eluate not subjected to gel filtration.

protein peak containing essentially pure γM resulted. Fractions 56 to 67 of the first P-300 column were found to consist of mixtures of all three immunoglobulin classes and were not further studied.

R.G.II eluate: An aliquot of this eluate, containing 985 μg N, was concentrated to 0.5 ml, layered on a 4 ml sucrose density gradient, and centrifuged for 16 hr at 35,000 RPM as previously outlined. Ten 0.45 ml fractions were collected and the immunoglobulin content of each fraction determined by agar diffusion (Fig. 1 E). Fractions 1 and 2 consisting almost exclusively of γM, and fractions 4 to 6 containing γG- and some γA-immunoglobulins were pooled. These 2 fractions, as well as fraction 3, which represented a mixture of γM, γG, and γA, were dialyzed thoroughly against buffered saline to remove sucrose. The immunochemical properties of these fractions, designated, as R.G. γM (tubes 1 and 2), γG (tubes 4 to 6), and intermediate (tube 3) were studied without further fractionation.

Table IV summarizes the analytical data on the purified fractionated antibodies and Fig. 2 depicts their precipitation curves with hog A substances. Total N recovery from the R.G.II eluate was 64% with 51% in the γG-fractions, 11.4% in the γM-fraction, and 1.5% in the intermediate fraction. From its absorbance at 2800 A, the intermediate Ortho fraction was estimated to contain 148 μg N; thus, an overall N yield of only 50% was achieved from the Ortho eluate. This comparatively poor recovery may be ascribed to protein denaturation due to prolonged exposure of the Ortho eluate to summer room temperatures (28–32°C) during gel filtration.

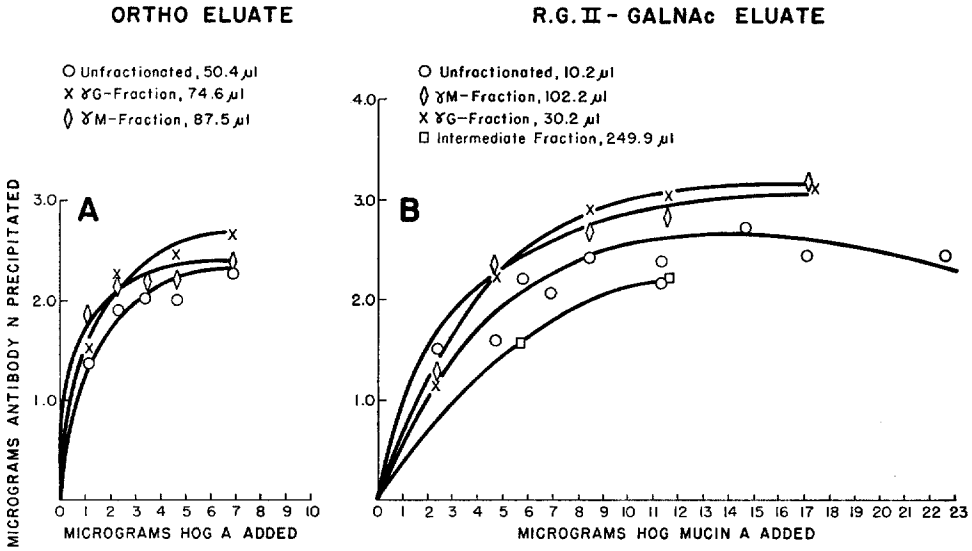


FIG. 2. Quantitative precipitin curves of whole anti-A eluates and their γM- and γG-fractions.

When the N content and absorbance at 2800 A of the individual antibody solutions are compared (Table IV, column 4), it is apparent that the value for OD/μg N obtained for the two γM-fractions deviate appreciably, and in opposite directions, from the γG-values.

Precipitability of the Ortho eluate and its fractions by hog A substance was rather low (43 to 52%) while that of the R.G.II eluate and its fractions was higher, ranging from 69% (γG) to 89% (γM). The A–anti-A precipitin curves for each eluate and its constituent immunoglobulin fractions (Fig. 2) were very similar and did not deviate in shape from the classical quantitative precipitin curve.

When hemagglutinating activity was expressed as the minimum amount of antibody in μg N/ml required to give detectable agglutination (Table IV, last

column), the R.G.II eluate was four times as active as the Ortho eluate, the R.G. γG-fraction seven times as active as the Ortho γG-fraction, while the activities of the two γM-fractions were essentially equal. The agglutinating activities of the Ortho antibody fractions were very similar. Much greater differ-

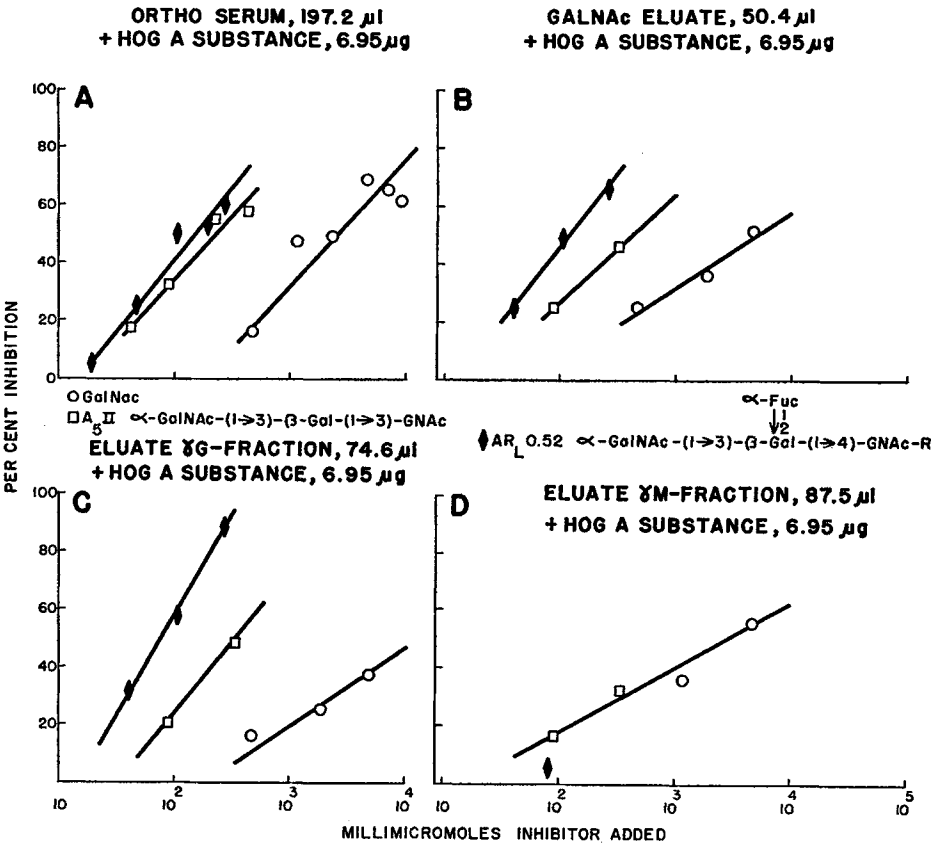


FIG. 3. Inhibition by oligosaccharides of the precipitation by A substance of Ortho anti-A and its purified antibody fractions.

ences were found with the various R.G. fractions (e.g. 0.43 µg N/ml of the R.G. γM-fraction as compared with 0.03 µg N/ml of the γG-fraction were required for detectable agglutination.)

Fig. 1 F demonstrates that the Ortho γM- and γG-, as well as the R.G. γM-, fractions contained both type K and L light chains. Although no effort was made to quantitate these determinants, the appearance of the various precipitin bands suggested that the two Ortho fractions contained a preponderance of type L molecules while type K molecules appeared in somewhat greater con-

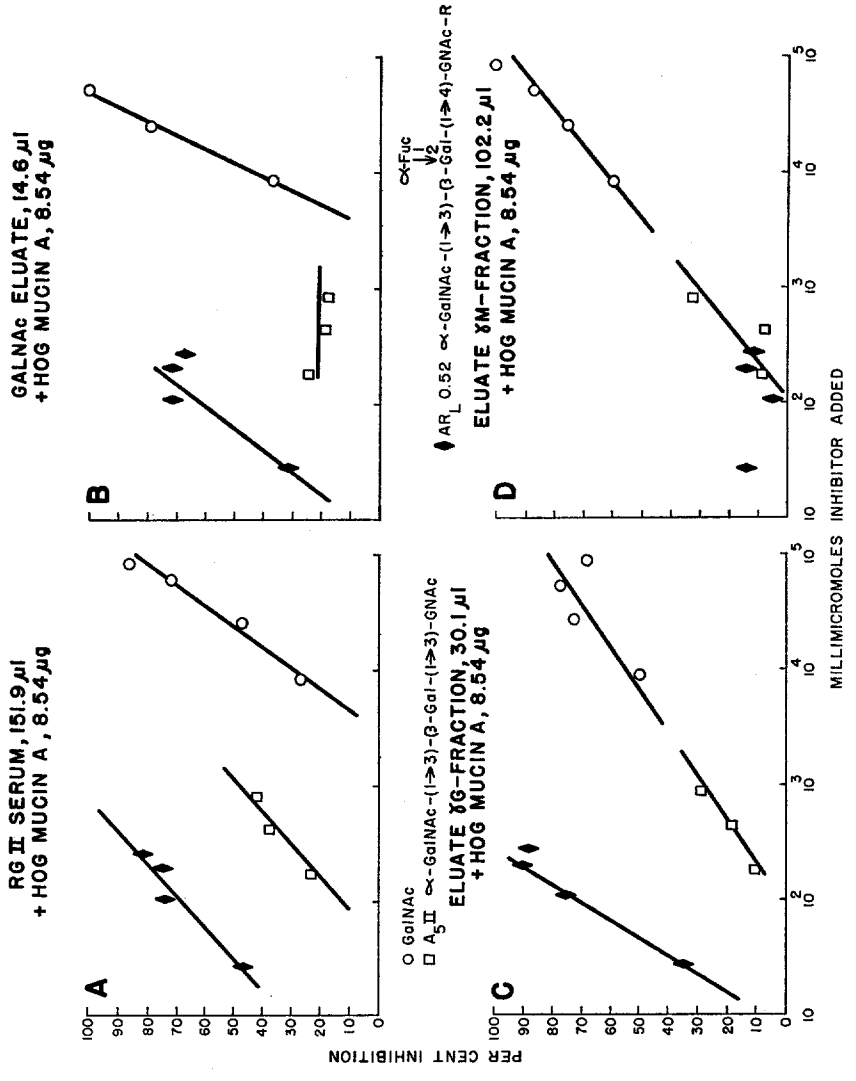


FIG. 4. Inhibition by oligosaccharides of the precipitation by A substance of R.G. anti-A serum and its purified antibody fractions.

centration in the R.G. γM-fraction. As seen in Fig. 1 F, the Ortho K-anti K lines and the L-anti L line developed by the R.G. γM fraction appeared relatively close to the antigen (i.e. immunoglobulin) wells and were quite broad and diffuse; the diffuseness was particularly striking on those sides of the precipitin bands facing the antigen wells. These findings are typical of immunodiffusion precipitin systems containing gross antibody excess (28, 29). The R.G. γG-fraction was found, in contrast, to be comprised almost exclusively of group K molecules. Only after prolonged incubation of the gel-diffusion plate did an extremely faint precipitin line develop between the R.G. γG-fraction and the anti-L serum. This line is not visible in Fig. 1 F.

TABLE V

Millimicromoles of Inhibitor Required for 50% Inhibition

Anti-A	Inhibitor		
	GalNAc	A5II (Trisaccharide)	AR_L 0.52 (Pentasaccharide)
Ortho			
Serum	2,600	230	150
Eluate	5,000	420	120
γG	13,000	350	76
γM	2,800	2800	?
R.G.II			
Serum	25,000	1200	33
Eluate	12,000	?	60
γG	4,700	4700	44
γM	4,100	4100	>300

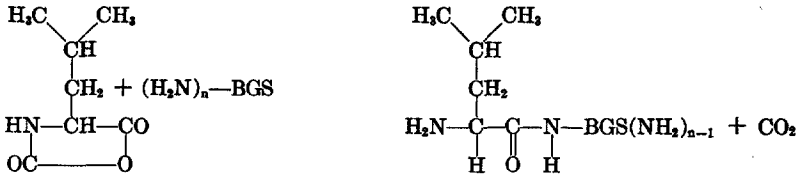
Precipitin-Inhibition Studies.—The behavior in quantitative hapten inhibition studies of the Ortho and R.G.II sera, the anti-A eluates and their γG- and γM-fractions is seen in Figs. 3 and 4 and is summarized in Table V. With both sera the pentasaccharide AR_L 0.52 was, on a molar basis, a far better inhibitor than the monosaccharide GalNAc; the trisaccharide (A5II) displayed intermediate activity. Similar results have previously been reported from this laboratory with other anti-A sera (17). Compared with serum R.G., the Ortho serum was considerably more inhibitable by GalNAc and A5II and slightly less inhibitable by AR_L 0.52. The unfractionated Ortho eluate was somewhat less inhibitable by GalNAc and more inhibitable by AR_L 0.52 than the original serum; the converse findings were noted in the case of serum R.G. and its eluate, i.e. the eluate was slightly more inhibitable by GalNAc and less inhibitable by AR_L 0.52 than the original serum. In relatively low concentrations, the pentasaccharide effectively inhibited the precipitation of both Ortho and R.G. γG-fractions (50% inhibition being achieved with 76 mμM and 44 mμM respectively).

In contrast, even at AR_L 0.52 concentrations of 270 mμM, less than 15% inhibition of precipitation of the γM-fractions could be achieved. Although inhibition points at higher oligosaccharide concentrations were not obtained because of limited material, the available data suggest that, on a molar basis (*a*) with both γM-anti-A fractions the penta- and trisaccharide were only as efficient as GalNAc in inhibiting precipitation, (*b*) with R.G. γG the trisaccharide and GalNAc were only $\frac{1}{1000}$ as active as the pentasaccharide in inhibiting precipitation (approximately 4700 mμM of the first two and 44 mμM of the last being required for 50% inhibition), and (*c*) with the Ortho γG–anti-A, the pentasaccharide was approximately 4 times as active as the trisaccharide which in turn was 40 times more active than GalNAc in inhibiting precipitation (50% inhibition points being obtained at concentrations of 76 mμM, 350 mμM, and 13,000 mμM respectively).

DISCUSSION

Antigens rendered insoluble by various means have been successfully employed as specific immune absorbants to purify homologous antibodies from serum (30, 31, cf. 18). Earlier efforts to isolate human blood group antibodies employing this technique were reported by Isliker (32) and by Goodman (33) who utilized red cell stroma coupled respectively to ion exchange resins and to polyurethane. Although they presented no data regarding the physicochemical properties of the recovered antibodies or the degree of purification achieved, this approach appeared to offer sufficient promise to merit further investigation especially in view of the successful use of Sephadex, an insoluble dextran, in purifying antidextran (25).

The studies herein reported clearly show that specific insoluble BGS are simply prepared by utilizing soluble blood group substances as multifunctional initiators for the polymerization of *N*-carboxy-L-leucine anhydride. The initial reaction is schematically depicted as follows:



Blood group substances, represented as BGS (NH_2) are mucopolysaccharides; the carbohydrate moieties of which contribute approximately 75 to 80% of the weight and antigenic specificity, are bound to peptides that are, apparently, immunologically inert (34). *N*-Carboxy-L-leucine anhydride reacts with free amino groups, present as the epsilon amino groups of lysine in the peptide portion of the BGS molecule, all of the amino sugars being *N*-acetylated. The

hypothetical intermediate product (*) resulting from the interaction of 1 molecule of carboxyanhydride with 1 molecule of BGS contains (n − 1) lysyl amino groups and a newly formed NH_2 group on the α-carbon of the attached leucine. Additional carboxyanhydride may then react at these sites to propagate the reaction. The final insoluble product is almost certainly heterogeneous, probably consisting of polyleucyl chains of various lengths attached to lysyl residues. If polymerization occurs as outlined, the primary structure of antigenically active sites should be undisturbed. The ability of these insoluble BGS to combine specifically with blood group antibodies (Table II) supports this concept.

The immunochemical properties of the various soluble blood group substances and of their polyleucyl derivatives were found to be very similar. Thus, McDon A and Pl-McDon A were capable of combining with less R.G. anti-A than were hog A or Pl-hog A. Such differences have been found with anti-A sera obtained from individuals hyperimmunized with hog A substances (11) but have not been seen in instances in which the antigenic stimulation was by heterospecific pregnancies as was the case with R.G.

The findings that Pl-A substance absorbed most of the anti-B while Pl-B removed very little of the anti-A from serum R.G. strongly suggested that much of the anti-B activity in the serum was due to cross-reacting antibody. Additional support for this conclusion was the observation that the R.G. antibodies obtained from Pl-B by galactose elution manifested as much anti-A as anti-B activity (Table III). The occurrence of cross-reacting antibodies in the serum of some type O individuals has been documented repeatedly (35, 36). Evidence suggests that the specificity of these antibodies is directed against structural features common to both blood group A and B oligosaccharide determinants (37).

The antibody yield resulting from acid elution of Pl-BGS (Table III) was poor; this is in agreement with the observations of Kochwa and Rosenfield (38) who recovered very little antibody when anti-A– or anti-B–sensitized red cell stroma were treated with pH 3.0 glycine buffer. In contrast, GalNAc anti-A eluates were found to contain as much as 70% of the total antibody N absorbed onto Pl-A. Antibody yields may be improved even further if larger haptens such as A5II or AR_L 0.52 were used for elution.

The presence of γM-, γG-, and γA-immunoglobulins in the Ortho and R.G. eluates confirms the observations of Kunkel and Rockey (8), Rawson and Abelson (9), and Ishizaka, et al. (10), that, in individual human sera, isohemagglutinins may occur in all 3 immunoglobulin classes. As previously described, the concentrated Ortho eluate contained an additional unidentified protein that migrated in the slow β-region on immunoelectrophoresis. Whether this represents doubling of the γM-precipitin line due to K and L determinants or an additional component could not be determined.

Except for the 19S γM-cold agglutinins, of which all reported examples have been type K (39, 40), a variety of purified human antibodies (γG as well as γM) have been found to contain both K and L light chain determinants in varying proportions. Thus, Mannik and Kunkel (41) observed that 6 purified anti-A and 2 anti-B antibodies exhibited both K and L determinants; of these antibodies 6 were primarily γG, one γM, and one γA. Similar findings have been reported for other human antibodies including anti-Rh (40, 41), antithyroglobulin (41), antiteichoic acid (41), and rheumatoid factors (39). The present studies differ from the previous ones in that antibodies of a given specificity were separated into γM- and γG-fractions and the L chain determinants of the latter compared. Both type K and L molecules (with a preponderance of L) were found in the two Ortho fractions. The R.G. γM-fraction also contained both light chain determinants and in this antibody fraction type K molecules appeared to predominate. In striking contrast, the R.G. γG-fraction was essentially devoid of type L determinants, being made up of type K molecules almost exclusively. Although this finding is of unusual interest, its significance cannot be fully assessed. It is generally believed that a single antibody-producing cell may synthesize either, but not both, type K or type L light chains (42). If, as proposed by Nossal et al. (43), individual antibody-forming cells synthesize first γM- then γG-antibodies of a given specificity, the antibodies produced, regardless of their configuration, would contain a relatively fixed proportion of K and L light chains. Since, the γM- and γG-immunoglobulins isolated from serum R.G. differed significantly in this respect, the following possibilities must be considered: (*a*) a selected population of γM-producing cells (principally L chain formers) failed to produce γG; (*b*) γG- and γM-antibodies of the same specificity are synthesized in different cells; or (*c*) the purification procedures employed resulted in the isolation of selected, phenotypically distinguishable populations of γM- and γG-immunoglobulins. The last alternative is discussed subsequently in another context.

The purified anti-A antibodies obtained from the Ortho and R.G. sera were incompletely precipitable by hog A substance (Tables III and IV). Similar findings have been reported previously with purified human antidextrans (25). Why the homologous antigens do not totally precipitate these purified antibodies is not understood. The problem in part involves difficulties in the analysis of very small amounts of antibody by the ninhydrin method (cf. 25, footnote to Table I). It is possible that such antigen-antibody complexes are unusually soluble or that the antibodies undergo structural changes during purification which render them less precipitable. The importance of solubility factors is emphasized by the results of the gel diffusion study illustrated in Fig. 1 D. The concentrated unfractionated, R.G. anti-A eluate placed in the center well was found, by quantitative precipitin analysis to be 71% precipitable by hog A substance (Table III) but the precipitin bands formed by the eluate and blood

group A substances were extensively penetrated by the γG–anti-γG, γM–anti-γM, and γA–anti-γA precipitin lines. This indicates that A-anti A precipitation in agar at 37°C is less complete than that occurring under conditions of the quantitative precipitin assay. The A-anti A complexes in agar at 37°C would appear to be significantly more soluble or dissociable than the immunoglobulin-anti-immunoglobulin precipitates.

The observations indicating that the purified γG–anti-A antibodies are, on a molar basis as active, if not more active, than γM-antibodies in agglutinating human type A_1 red cells (Table IV) are at variance with previously published studies (10, 44). Ishizaka, et al. (10), measuring specific antibody content by indirect methods, estimate that the minimum levels of human anti-A in immunoglobulin concentrates required to induce hemagglutination are, for γM, 0.0004 to 0.0008 μg AbN/ml, and for γG, 0.01 μg AbN/ml, AbN concentrations being determined by uptake of radioactive iodine. The present studies suggest minimal hemagglutinating concentrations of 0.39 and 0.43 μg N/ml for γM and 0.03 and 0.21 μg N/ml for γG–anti-A antibodies respectively. While the data for γG-antibodies are in substantial agreement, those for the γM-antibodies differ by a factor of 1000. The reasons for this are not immediately apparent. It is possible that GalNAc elutes only weakly binding, poorly agglutinating γM–anti-A from Pl-A, while the more strongly binding, actively agglutinating γM-molecules resist elution and are not recovered or studied.

The capacity of haptens of various sizes to inhibit precipitation of antibody by homologous antigen (quantitative hapten inhibition technique) is believed to reflect the degree to which the haptens conform to the antibody-combining site (cf. 18). Thus, after it was demonstrated with several antidextran sera having 1, 6 specificity that isomaltohexaose or isomaltoheptaose were, on a molar basis, the most efficient inhibitors of dextran-antidextran precipitation, it was inferred that the maximum size of the antibody combining site was in the range of a hexa- or heptasaccharide (45).

By performing such studies with γM– and γG–anti-A antibodies isolated from individual sera it was hoped to elucidate and compare the combining site characteristics of these two antibody classes. It was found that the precipitation of γG–anti-A fractions was most effectively inhibited by the pentasaccharide hapten AR_L 0.52 while the γM-antibodies from the same sera were inhibited to the same degree by equimolar concentrations of monosaccharide (GalNAc), trisaccharide, and pentasaccharide haptens. These findings suggest that the antibody-combining sites of these isolated immunoglobulin fractions differ, the γG-sites being sufficiently large to accommodate, at least, a pentasaccharide while the capacity of the smaller γM-sites is limited to GalNAc, the terminal nonreducing sugar of the A-antigenic determinant.

The question arises as to whether the small combining site size of the γM–anti-A is characteristic of all γM–anti-A molecules or whether the method of

isolation involves some selection of molecules with smaller size combining sites. Some evidence of selection is apparent in comparing the whole serum with the eluates as well as with the separated γM- and γG-fractions (Figs. 2 and 3). Since the available data indicates that γM-antibodies have a valence of five or six (46, 47) while γG-antibodies are bivalent, it is quite possible that the GalNAc extraction of the polyleucyl BGS–anti-A precipitate eluted only γM-antibodies with small combining sites, e.g. complementary to GalNAc, while simultaneously extracting a more representative sample of the γG–anti-A. This, as noted above, could account for the observed differences in hemagglutinating potency for the γM–anti-A in this and other laboratories. Further information on this point will become available when extracts are made with larger haptens such as the pentasaccharide.

Evidence that combining site heterogeneity does, in fact, exist among the γG–anti-A immunoglobulin molecules present in an individual serum is seen in Figs. 3 and 4. A5II, the trisaccharide hapten, inhibited precipitation of the Ortho γG-fraction to a degree intermediate between GalNAc and the pentasaccharide hapten AR_L 0.52. This suggests that more γG-molecules are able to react with the trisaccharide than with GalNAc alone and that when fucose is linked to the subterminal galactose of the trisaccharide (forming a branched oligosaccharide), antibody binding is again substantially increased. With respect to the R.G. γG-fraction, in contrast, the trisaccharide hapten was no more inhibitory than was GalNAc. These differences are quite consistent with earlier interpretations (cf. 17, 48) of the role of the fucose side chain on the subterminal galactose of AR_L 0.52 in increasing the inhibiting power of the trisaccharide hapten by stabilizing a preferred conformation of the A determinant. Evidence for preferred conformations of oligosaccharides containing GNAc and GalNAc in solution has been obtained by optical rotatory dispersion studies (48).

SUMMARY

Insoluble blood group substances prepared by copolymerization of soluble blood group substances with *N*-carboxy-L-leucine anhydride were used to absorb blood group antibodies from two human, high-titered anti-A sera. After the absorbants were washed free of nonspecific serum proteins, blood group antibodies were eluted either with pH 3.62 acetate buffer, or at neutral pH with monosaccharide haptens of the A or B antigenic determinants (*N*-acetyl-D-galactosamine or D-galactose respectively). The purified anti-A antibodies were characterized, immunoelectrophoretically, as γM-, γA-, and γG-immunoglobulins. These were further separated into γM- and γG-fractions by gel filtration or density gradient centrifugation. Both γM- and one of the two γG-antibody fractions contained K and L light chain determinants; the remaining γG-fraction was comprised, almost totally, of type K molecules.

Precipitability of the purified anti-A immunoglobulins by blood group A substance varied from 43 to 89%. The agglutinating activity per unit N of the isolated γG–anti-A was found to equal, in one case, and to exceed, in the second, that of the γM-antibodies from the same individuals.

The marked differences between γM- and γG-antibody fractions in quantitative hapten inhibition studies were interpreted to mean that the antibody-combining site of the isolated eluted γG–anti-A was significantly larger than that of the eluted γM–anti-A. Whether these data connote differences in combining site size between entire immunoglobulin classes in an individual serum or simply reflect the properties of highly selected antibody populations cannot be stated at present.

BIBLIOGRAPHY

1. Heremans, J., Les Globulines Sériques du Système Gamma, Leur Nature et leur Pathologie, Paris, Masson, 1960.
2. Cohen, S., and Porter, R. R., Structure and biological activity of immunoglobulins, *Advances Immunol.*, 1964, **4,** 287.
3. Kabat, E. A., Structure and heterogeneity of antibodies, *Acta Hematol.*, 1966, **36,** in press.
4. World Health Organization, Nomenclature for human immunoglobulins, *Bull. World Health Organ.*, 1964, **30,** 447.
5. Rowe, D. S., and Fahey, J. L., A new class of human immunoglobulins. I. A unique myeloma protein, *J. Exp. Med.*, 1965, **121,** 171.
6. Fahey, J. L., personal communication.
7. McDuffie, F. M., Allen, P. Z., Kabat, E. A., and Williams, C. A., Jr., An immunochemical study of the relationship of human blood group isoantibodies to γ1 and γ2 globulins, *J. Immunol.*, 1958, **81,** 48.
8. Kunkel, H. G., and Rockey, J. H., β_2A and other immunoglobulins in isolated anti-A antibodies, *Proc. Soc. Exp. Biol. and Med.*, 1963, **113,** 278.
9. Rawson, A. J., and Abelson, N. M., Studies of blood group antibodies. VI. The blood group isoantibody activity of γ_1A globulin, *J. Immunol.*, 1964, **93,** 192.
10. Ishizaka, K., Ishizaka, T., Lee, E. H., and Fudenberg, H., Immunochemical properties of human γA isohemagglutinins. I. Comparisons with γG- and γM-globulin antibodies, *J. Immunol.*, 1965, **95,** 197.
11. Kabat, E. A., Blood Group Substances, New York, Academic Press, 1956.
12. Tsuyuki, H., Van Kley, H., and Stahmann, M. A., The preparation and physical properties of polypeptidyl proteins, *J. Am. Chem. Soc.*, 1956, **78,** 764.
13. Schiffman, G., Kabat, E. A., and Leskowitz, S., Immunochemical studies on blood groups. XXVI. The isolation of oligosaccharides from human ovarian blood group A substance, including two disaccharides and a trisaccharide involved in the specificity of the blood group A antigenic determinant, *J. Am. Chem. Soc.*, 1962, **84,** 73.
14. Schiffman, G., Kabat, E. A., and Thompson, W., Immunochemical studies on blood groups. XXX. Cleavage of A, B, and H blood group substances by alkali, *Biochemistry*, 1964, **3,** 113.
15. Lloyd, K. O., and Kabat, E. A., Some products of the degradation of blood group

substances by alkaline borohydride, *Biochem. and Biophysic. Research Commun.*, 1964, **5,** 385.

16. Lloyd, K. O., Kabat, E. A., Layug, E. J., and Gruezo, F., Immunochemical studies on blood groups. XXXIV. Structures of some oligosaccharides produced by alkaline degradation of blood group A, B, and H substances, *Biochemistry*, 1966, **5,** 1489.
17. Lloyd, K. O., Kabat, E. A., and Rosenfield, R. E., Immunochemical studies on blood groups. XXXV. The activity of fucose containing oligosaccharides isolated from blood group A, B and H substances by alkaline degradation, *Biochemistry*, 1966, **5,** 1502.
18. Kabat, E. A., Kabat and Mayer's Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, Publisher, 2nd edition, 1961.
19. Kabat, E. A., and Leskowitz, S., Immunochemical studies on blood groups. XVII. Structural units involved in blood group specificity, *J. Am. Chem. Soc.*, 1955, **77,** 5159.
20. Rosevear, J. W., and Smith, E. L., Glycopeptides. I. Isolation and properties of glycopeptides from a fraction of human γ-globulin, *J. Biol. Chem.*, 1961, **236,** 425.
21. Kunkel, H. G., Macroglobulins and high molecular weight antibodies, *in* The Plasma Proteins, (Putnam, F. W., editor) New York, Academic Press, 1959, 225.
22. Ouchterlony, O., Antigen-antibody reactions in gels, *Acta Path. et Microbiol. Scand.*, 1948, **25,** 186.
23. Halbert, S. P., Swick, L., and Sonn, C., The use of precipitin analyses in agar for the study of human streptococcal infections. II. Ouchterlony and Oakley techniques, *J. Exp. Med.*, 1955, **101,** 557.
24. Grabar, P., and Williams, C. A., Jr., Méthode permettant, l'etude conjuge des propriétés électrophorétiques et immunochemiques d'un mélange de protéines. Application au sérum sanguin, *Biochim. et Biophysica Acta*, 1953, **10,** 193.
25. Schlossman, S. F., and Kabat, E. A., Specific fractionation of a population of antidextran molecules with combining sites of various sizes, *J. Exp. Med.*, 1962, **116,** 535.
26. Kunkel, H. G., Mannik, M., and Williams, R. C., Individual specificity of isolated antibodies, *Science*, 1963, **140,** 1218.
27. Watkins, W. M., and Morgan, W. T. J., Inhibition by simple sugars of enzymes which decompose the blood group substances, *Nature*, 1955, **175,** 676.
28. Oudin, J., Specific precipitation in gels and its application to immunochemical analysis, *Methods Med. Research*, 1952, **5,** 335.
29. Crowle, A. J., Immunodiffusion, New York, Academic Press, 1961.
30. Campbell, D. H., Luescher, E., and Lerman, L. S., The use of an immunologically specific adsorbant for purification of antibody, *Proc. Nat. Acad. Sc.*, 1951, **37,** 575.
31. Isliker, H. C., The chemical nature of antibodies, *Advances Protein Chem.*, 1957, **12,** 387.
32. Isliker, H. C., Purification of antibodies by means of antigens linked to ion exchange resins, *Ann. New York Acad. Sc.*, 1953, **57,** 225.

33. Goodman, H. S., Chromatographic fractionation and characterization of antibody based on the thermal dissociation of the specific antigen-antibody complex, *Nature*, 1962, **193**, 1.
34. Carsten, M. E., and Kabat, E. A., Immunochemical studies on blood groups. XIX. The amino acids of blood group substances, *J. Am. Chem. Soc.*, 1956, **78**, 3083.
35. Landsteiner, K., and Witt, D. H., Observations on the human blood groups. Irregular reactions. Isoagglutinins in sera of group 4. The factor A1, *J. Immunol.*, 1926, **11**, 221.
36. Race, R. R., and Sanger, R., Blood Groups in Man, Oxford, Blackwell Scientific Publication, 1962.
37. Schiffman, G., and Howe, C., The specificity of blood group A-B cross-reacting antibody, *J. Immunol.*, 1965, **94**, 197.
38. Kochwa, S., and Rosenfield, R. E., Immunochemical studies of the Rh system. I. Isolation and characterization of antibodies, *J. Immunol.*, 1964, **92**, 682.
39. Franklin, E. C., and Fudenberg, H. H., Antigenic heterogeneity of human Rh antibodies, rheumatoid factors, and cold agglutinins, *Arch Biochem. and Biophysics*, 1964, **3**, 433.
40. Leddy, J. P., and Bakemeier, R. F., Structural aspects of human erythrocyte autoantibodies, *J. Exp. Med.*, 1965, **121**, 1.
41. Mannik, M., and Kunkel, H. G., Localization of antibodies in group I and group II γ-globulins, *J. Exp. Med.*, 1963, **118**, 817.
42. Bernier, G. M., and Cebra, J. J., Polypeptide chains of human gamma globulin. Cellular localization by fluorescent antibody, *Science*, 1964, **144**, 1590.
43. Nossal, G. J. V., Szenberg, A., Ada, G. L., and Austin, C., Single cell studies on 19S antibody production, *J. Exp. Med.*, 1964, **119**, 485.
44. Greenbury, C. L., Moore, D. H., and Nunn, L. A. C., Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells, *Immunology*, 1963, **6**, 421.
45. Kabat, E. A., The upper limit for the size of the human antidextran combining site, *J. Immunol.*, 1960, **84**, 82.
46. Onoue, K., Yagi, Y., Grossberg, A. L., and Pressman, D., Number of binding sites of rabbit macroglobulin antibody and its subunits, *Immunochemistry*, 1965, **2**, 401.
47. Humphrey, J. H., personal communication.
48. Beychok, S., and Kabat, E. A., Optical Activity and conformation of carbohydrates. I. Optical rotatory dispersion studies on immunochemically reactive amino sugars, and their glycosides, milk oligosaccharides, oligosaccharides of glucose, and blood group substances, *Biochemistry*, 1965, **4**, 2565.