STUDIES ON HUMAN ANTIBODIES

VIII. Properties and Association Constants of Human Antibodies to Blood Group A Substance Purified with Insoluble Specific Adsorbents and Fractionally Eluted with Mono- and Oligosaccharide*

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Antibodies to the blood group A and B substances in human sera are extraordinarily heterogeneous. Sera generally contain γG, γM, and γA anti-A or anti-B (1–6). Differences in properties of various sera by precipitation (4, 5), hemagglutination (4, 5, 7–9), hemolysis (8, 10, 11), complement fixation, (10–12) and fixation to skin (4, 13) have been found. To understand the extent of this heterogeneity it is essential to prepare and study blood group antibodies of the various immunoglobulin classes from single donors. In an earlier study from this laboratory (5), insoluble blood group A and B substances were prepared by attaching polyleucyl side chains to them, using the *N*-carboxyanhydride method (14). Large volumes of anti-A could be specifically absorbed on these products and, after washing to remove nonspecific protein, a considerable proportion of the antibody was eluted with *N*-acetylgalactosamine (GalNAc). This eluate could be fractionated into γM anti-A and γG anti-A; these two anti-A fractions showed striking differences with respect to the relative capacities of mono- and oligosaccharides to inhibit precipitation by antigen. With the γG anti-A, a reduced pentasaccharide was a much better inhibitor on a molar basis than a trisaccharide which in turn was better than *N*-acetylgalactosamine, while with the γM anti-A, all three compounds were equally potent (5).

The present study extends these observations, adding the characterization of a second population of antibodies obtained by eluting with a specific blood group A pentasaccharide ($AR_L0.52$) the anti-A antibodies which had remained absorbed on the insoluble antigen after elution with *N*-acetylgalactosamine.

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Studies by inhibition of precipitation have been correlated with association constants determined by equilibrium dialysis. Other properties of the various antibody fractions including hemagglutination, complement fixation, hemolytic activity, and kinds of light chains are also reported.

Materials and Methods

Antisera—Subject 1 (blood group O+): Received two immunizations of 1 mg hog mucin blood group A + H substance 1 day apart in 1945, and a second course of 2 mg, 5 months later (15). A very slow decrease in anti-A titer has been observed through all these years (16). Two consecutive bleedings, D_{33} and D_{34}, taken in February 1963 were pooled and studied. Subjects, Jos. (B+) and Chris. (O+), were immunized with two injections of 1 mg commercial hog mucin A blood group substance (Pfizer Diagnostics, New York).[1] Two bleedings were obtained from each donor.

Anti-A serum 59-113 (17) with high agglutinating titer, was used routinely for inhibition of agglutination. Eel anti-H serum (18), a gift from Dr. Georg Springer, Northwestern University, and seed extract from *Ulex europeus* (19) were used to test H activity by inhibition of agglutination. Rabbit and goat antisera to human γG, γM, and γA immunoglobulins and to whole human serum were purchased from Hyland Laboratories, Los Angeles, Calif., and from Lloyd Brothers, Inc., Cincinnati, Ohio. Rabbit anti-human κ and λ chain sera were donated by Dr. Henry G. Kunkel of Rockefeller University.

Blood Group Substances (BGS)—Hog mucin blood group substance with A and H activities was purified from commercial hog gastric mucin (Wilson Laboratories, Chicago, Ill.) by ethanol precipitation, as previously described (20).

Insoluble polyleucyl blood group A substance (PLA) was prepared from the purified hog mucin in BGS by coupling with *N*-carboxyleucylanhydride (Pilot Chemicals Inc., Watertown, Mass.) (5). Human A substance (MSM) has been described previously (21).

Carbohydrates—*N*-acetyl-D-galactosamine (GalNAc) and *N*-acetyl-D-glucosamine (GNAc) were purchased from Nutritional Biochemical Corp., Cleveland. L-fucose (Fuc) and D-galactose (Gal) were from Mann Research Laboratories, New York. A_5II, a trisaccharide with A activity was isolated earlier from acid hydrolysates of BGS (17). Its structure is:

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

$AR_I0.52$ pentasaccharide, also A active, has been described (22, 23). It has the structure:

$$\begin{array}{c} \alpha\text{-L-Fuc } 1 \\ \downarrow \\ 2 \\ \alpha\text{-D-GalNAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal-}(1 \rightarrow 4)\text{-}\beta\text{-D-GNAc-}(1 \rightarrow 6)\text{-}R \end{array}$$

in which R represents a mixture of hexenetetrols $CH_2OH—CHOH—CH=CH—CHOH—CH_2OH$ formed by alkaline borohydride degradation from a Gal residue (24). This pentasaccharide was purified according to the following procedure: 28 g of hog mucin blood group substance (A + H active) were added to 280 ml of a 0.2 N NaOH–1% sodium borohydride solution. The material went into solution and was stirred for 2 days at 4°C and for 5 days at room temperature. After neutralization the product was dialyzed, the dialysate concentrated by evaporation at reduced pressure and desalted by adding 320 g of mixed resin (Amberlite MB 3). The desalted dialysate (14.5 g) was fractionated in a charcoal-celite column (320 mm ×

[1] We thank Dr. R. E. Rosenfield, Mount Sinai Hospital, New York, for his collaboration in immunizing these volunteers.

80 mm) containing 350 g charcoal and 175 g celite (25). The column was eluted with water and then with an ethanol gradient 0–70% ethanol.

Oligosaccharides were located by periodate uptake and paper chromatography (23).

$AR_L0.52$ is eluted at an ethanol concentration of approximately 15%, but location of the peak by periodate uptake is anomalous since on oxidation it gives products absorbing at 225 mμ[2] and the phenol–sulfuric acid method (26) must be used.

The fraction rich in $AR_L0.52$, 2.04 g, was applied to 15 sheets of chromatographic paper (S and S paper No. 589 Green Label) and run for 20 hr in butanol, pyridine, water, 6:4:3. The papers were cut as determined from small strips. Oligosaccharides were eluted with water, lyophilized, and chromatographed on a Bio-Gel P2 column (820 mm × 19 mm), eluting with water. A small shoulder of a slower-moving compound was eliminated and those fractions showing only one component, $AR_L0.52$, by paper chromatography were pooled and analyzed. This fraction, $AR_L0.52$ (345 mg) was then repurified on a charcoal-celite column (450 mm × 28 mm) as above. The main peak, containing the pentasaccharide $AR_L0.52$ (197 mg) gave a single spot on paper chromatography and analysis of sugars as expected from the formula. This material was used to elute anti-A antibodies from the columns of PLA-BGS and to pre-

TABLE I

Analytical Properties of ^3H–$AR_L0.52$ Pentasaccharide

	Total N	Methyl-pentose	Galactose	Hexosamine	NAc hex-osamine	Molar ratio $\frac{\text{NAc hexosamine}}{\text{hexosamine}}$
	%	%	%	%	%	
Found	3.3	18.3	20.8	37.8	30.2	0.65
Expected	3.2	19.2	20.9	41.5		0.65

pare tritiated $AR_L0.52$; the $AR_L0.52$ was recovered and repurified on a charcoal-celite column after each experiment.

Labeling of $AR_L0.52$—This was accomplished by taking advantage of the unsaturated character of the residue (R) at the reducing end of $AR_L0.52$. 100 mg of $AR_L0.52$ were sent to New England Nuclear Corp., Boston, Mass., to be reduced catalytically in the presence of 3c of tritium gas. The compound was purified in this laboratory by the following procedure.

One-half of the sample was passed through a column of Bio-Gel P 2 (770 mm × 19 mm). Although a single, rather symmetrical radioactive peak was obtained, the nonconstant ratio of radioactivity to sugar content (estimated by the phenol-sulfuric method) suggested the presence of several components. This was confirmed by paper chromatograms and locating the radioactivity in a Packard scanner type counter, equipped with gas-flow counter and open window.[3] Therefore, the sample was purified twice by preparative paper chromatography as described above for the nonradioactive compound $AR_L0.52$. The product was then repurified passing it through a Bio-Gel P 2 column. The single sharp peak from the column gave a single radioactive peak (97% of total area) on paper chromatograms with a mobility exactly the same as the nonradioactive $AR_L0.52$, and gave satisfactory analytical values and was used for all the studies. (Table I).

The other half of the sample from New England Nuclear Corporation was purified passing

[2] Moreno, Carlos, and Elvin A. Kabat. Unpublished observation.

[3] We thank Dr. R. Canfield for allowing us to use this apparatus.

it four times through a charcoal-celite column to give a compound with analytical values equivalent to the other portion. From 100 mg $AR_L0.52$, a total of 11.6 mg of ^3H–$AR_L0.52$ were obtained with a specific activity of 6.89×10^8 cpm/μmole.

Analytical Methods—The following methods were used: Total nitrogen (21); periodate uptake (27); phenol-sulfuric reaction (26). Hexosamine, NAc hexosamine, methylpentose, and hexose (galactose) determination were performed as described in (27).

Paper Chromatography—Descending paper chromatography on Schleicher and Schuell No. 589 green ribbon was used analytically and preparatively with butanol–pyridine–water (6:4:3) as eluant (23).

Density Gradient Centrifugation—Linear 10–40% sucrose gradients in saline–phosphate buffer were used to separate γG from γM antibodies (28). An SW 25.1 rotor with adaptors was used for small volumes (33 hr, 22,500 rpm) and an SW 41 (24 hr, 30,000 rpm) when larger volumes were centrifuged. Density gradients were similar to those described in reference 5.

Assays for Tritium—Counting of radioactive samples was done in a Packard Tri-Carb liquid scintillation counter using 10 ml of Bray's solution per vial (29). Light activation was avoided and an external standard was used to estimate the amount of quenching in each sample. Blanks and reference solutions with known amounts of radioactivity were included. Each sample was always counted twice,[4] often in two different counters.

Immunochemical Methods—Quantitative precipitin assays and quantitative inhibition assays were performed as described (17). Hemagglutination and hemagglutination inhibition were carried out using Takatsy microtitrators (Cooke Engineering Co., Alexandria, Va.) (5). Titrations of antibody fractions from a given serum were performed simultaneously. A micromethod for double diffusion in agar was used (27). Quantitative radial immunodiffusion (30, 31) using immunoplates (Hyland Laboratories) was employed to estimate the γG, γA, and γM content of immunoglobulin fractions. Purified immunoglobulins were used as standards and several concentrations of unknown and standard were included in each plate. Estimation of immunoglobulin content was made graphically by plotting $(\text{radius})^2$ vs. concentration.

Immunoelectrophoresis—Performed according to Grabar and Williams (32).

Hemolysis—Hemolysis assays were done as described in (33).

C′ Fixation—The method of Wasserman and Levine (34) was used. C′ fixation was performed only with purified antibody. Incubation time for fixation was 30 min at 37°C and overnight at 4°C; $2C'H_{50}$ units per tube were used, enough to reach approximately 90% lysis in the control tube. Guinea pig C′, sheep red cells, and hemolysin were purchased from Baltimore Biological Laboratories, Baltimore, Maryland.

Equilibrium Dialysis—This procedure was carried out using glass cells manufactured by Buchler Instruments. Fort Lee, N.J. Incubation for 80 hr at 25°C was required for equilibrium. The method as well as the description of the cells can be found in (27). The total volume per chamber was 1 ml. Aliquots from both sides, in duplicate, were taken and counted.

Purification of Antibodies—As described previously (5), purification of antibodies was done with insoluble polyleucyl blood group A + H substance as immunoadsorbent. After centrifugation at 2000 rpm for several days, the clarified serum was passed through a small column of PLA mixed with celite in a ratio of 1:6. Absorption of antibodies was followed by agglutination titer of the effluent. The entire procedure was carried out at 4°C. A tendency of the column to stop flowing was noticed if the column was not wide enough (ratio length to diameter should be <6) and if analytical grade celite was used to prepare the column. After absorption of antibodies, the column was extensively washed with saline buffer pH 7.2, until practically no material absorbing at 280 mμ was detected in the washings.

Elution with GalNAc was then performed by letting a 10% solution of GalNAc (in saline buffer) penetrate the column and incubating for 1 hr at 37°C. The column was drained, washed with saline buffer at 4°C, the eluate concentrated by ultrafiltration (Schleicher and Schuell

[4] We thank Dr. H. Rosenkranz and Dr. P. Marks for the use of their counters.

collodion bags) under reduced pressure and then passed through a column of Bio-Gel P 10 (820 mm × 19 mm) to eliminate GalNAc. Elution with GalNAc under the same conditions was repeated until essentially no (or very little) antibody was recovered, indicating that antibody capable of being eluted with the GalNAc had already been removed. All antibodies eluted with GalNAc from a given column were pooled and will be referred to as GalNAc eluates.

After this extensive extraction with GalNAc, $AR_L0.52$ pentasaccharide was used to elute (the amounts will be given for each experiment) the remaining antibody from the column. Incubation was at 37°C for 1 hr and the eluates were concentrated in the same way. The limited amount of $AR_L0.52$ available did not permit successive elutions, and therefore, the first 10 ml of ultrafiltrate obtained during the concentration of the first eluate with $AR_L0.52$ were used for a second extraction under the same conditions. The two eluates were pooled, concentrated, and dialyzed. Passage twice through a column of Bio-Gel P 10 (820 mm × 19 mm) eliminated all the residual $AR_L0.52$ bound to the antibody as demonstrated by absence of sugar in the included volume of the column.

RESULTS

The procedure used to fractionate the antibodies from the whole serum is illustrated in Fig. 1. The same procedure was used with all three of the six anti-A sera fractionated, except for the last elution at high concentrations of salt. For serum 1, 2 M NaI was used and for serum Jos., successive elutions with 0.5, 1, 2, and 4 M KCNS were used (35, 36). Although all the eluates obtained with NaI and KCNS contained some small amount of protein, none had more than traces of agglutinating activity. No elution at high salt concentration was attempted with Chris. antiserum.

The yields of antibody absorbed on the column, the amount of hapten used to elute and the recoveries in each eluate are given in Table II. The efficiency of the absorption was measured by the amount of precipitating antibody remaining in the antiserum after passing it through the column. With serum 1, which had only 86% of the antibody absorbed, the 14% remaining was easily taken out by passing the serum through a second column of PLA. The absorption from antisera Jos. and Chris. was 97 and 100% respectively.

The amount of GalNAc used for elution, as indicated in Table II, was the quantity needed to take out all the antibody capable of being eluted by several additions of this sugar. The amount of pentasaccharide $AR_L0.52$ used for elution was determined by the amount available at the time of the experiment. With respect to recovery of purified antibody, Table II gives two values for the antibody content of antiserum 1, one obtained with hog, and the other, with human blood group A substance. This is because, while this work was under way, it was found that this particular antiserum had antibodies to a "non-A" determinant present in hog, but not in the human, blood group materials. This antibody amounts to approximately 30% of the total precipitated by hog substance[5] and it all remained absorbed in the column. The estimate of the total

[5] Moreno, C., and E. A. Kabat. 1969. Immunochemical studies on blood groups. XLIV. Human antibodies against a new determinant present in blood group substance from hog gastric mucin. *J. Immunol.* In press.

amount of antibody recovered by elution from the column was therefore calculated from the human blood group substance assays. The total yields of antibody ranged from 72% for serum 1 to 49% for serum Jos., and there were sub-

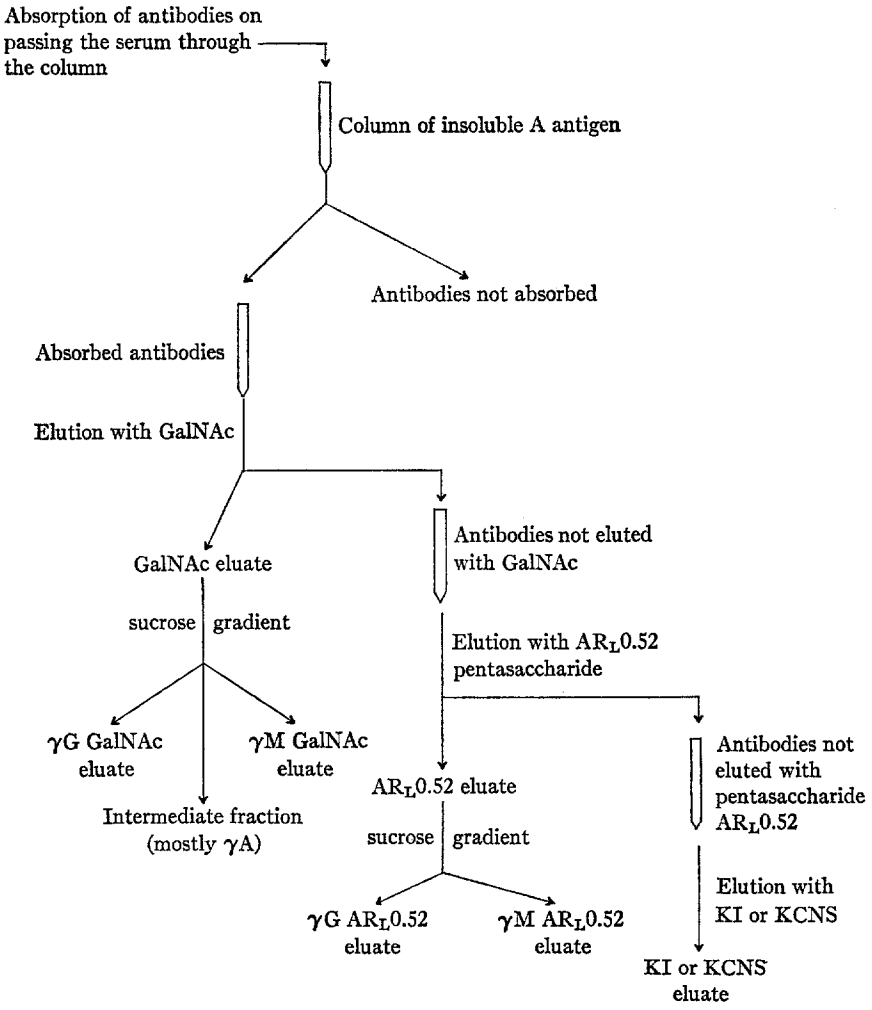

FIG. 1. Scheme of antibody fractionation.

stantial differences among the sera with respect to the amount of antibody obtained in each eluate. For sera 1 and Jos., the bulk of the antibody was obtained in the GalNAc eluate. For serum Chris., the yield of antibody obtained in the $AR_L0.52$ eluate (31%) was very close to that in the GalNAc eluate (34%).

The composition of the GalNAc and $AR_L0.52$ eluates, listed in Table III, reveals that although all three sera contained γG, γA, and γM antibodies, their relative concentrations varied substantially. The GalNAc eluate of serum 1 was very rich in γM (46%), but this immunoglobulin was practically absent from the $AR_L0.52$ eluate of the same serum. With both serum 1 and Jos., practically all the γM antibody was present in the GalNAc eluate; the $AR_L0.52$ eluates of

TABLE II

Recoveries of Antibody in Fractions Eluted from PLA Columns

Serum	Volume	Antibody concentration in serum	Amount of PLA used	Antibody absorbed	Amount of GalNAc used to elute	Recovery of antibody in GalNAc eluate	Amount of $AR_L0.52$ pentasaccharide used to elute	Recovery of antibody in $AR_L0.52$ eluate	Total recovery
	ml	μg N/ml	*mg*	%	*g*	*% of absorbed*	*mg*	%	%
No. 1	1,084	9.0*	576	86	7	38*	200‡	11*	49*
		6.1§				56§		16§	72§
Jos.	233	65.5	400	96	5	35	170‡	14	49
Chris.	222	38.8	400	100	2	34	80‡	31	65

* Tested with hog A + H blood group substance.

‡ Passed twice through the column.

§ Tested with human A (MSM) blood group substance.

TABLE III

Composition of the Eluates Obtained from PLA Columns

Serum	GalNAc eluate			$AR_L0.52$ eluate		
	γG	γA	γM	γG	γA	γM
	%	%	%	%	%	%
No. 1	46	12	46	100	traces	traces
Jos.	94	traces	14	100	traces	traces
Chris.	79	traces	29	89	traces	18

these two sera contained almost exclusively γG. With serum Chris., 18% of the $AR_L0.52$ was γM, a quantity not much lower than the 29% of γM obtained in the GalNAc eluate of the same serum. The GalNAc eluate of serum 1 differed from the corresponding eluate of the other sera in two respects: the γM content was very high (46%) and it was the only one in which there was enough γA for quantitative estimation.

Figs. 2 *a*, 2 *b*, and 2 *c* present the results of quantitative precipitin studies with antisera 1, Jos., and Chris., respectively. Except for the γG GalNAc eluate

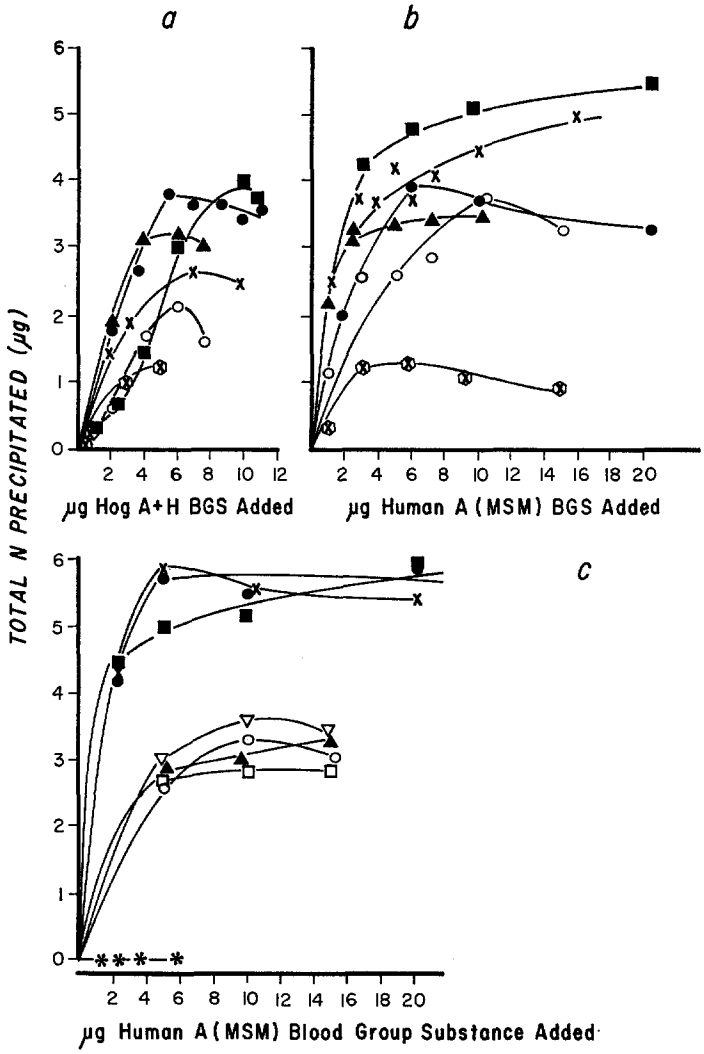

FIG. 2. Quantitative precipitin curves of anti-A sera and purified anti-A antibodies.

(*a*). Serum No. 1. ×, 297 μl unabsorbed serum; ⊗, 1.0 ml absorbed serum (PLA); ●, 5.46 μg N GalNAc eluate; ○, 5.40 μg N γG GalNAc eluate; ▲, 4.75 μg N γM GalNAc eluate; ■, 6.80 μg N γG AR_L0.52 eluate.

(*b*). Serum Jos. ×, 74.7 μl unabsorbed serum; ⊗, 0.494 μl absorbed serum (PLA); ●, 5.55 μg N GalNAc eluate; ○, 6.30 μg N γG GalNAc eluate; ▲, 5.05 μg N γM GalNAc eluate; ■, 5.45 μg N γG AR_L0.52 eluate.

(*c*). Serum Chris. ×, 150.3 μl unabsorbed serum; ✳, 1.0 ml absorbed serum (PLA); ●, 7.88 μg N GalNAc eluate; ■, 6.44 μg N AR_L0.52 eluate; ○, 4.40 μg N γG GalNAc eluate; ▲, 4.16 μg N γM GalNAc eluate; ▽, 4.20 μg N γG AR_L0.52 eluate; □, 3.86 μg N γM AR_L0.52 eluate.

of serum 1 (Fig. 2 *a*) all the sera and eluates that were studied in the zone of antigen excess did not show substantial solubility of the complex in moderate antigen excess. The point of maximum precipitation was used to estimate the percentage of precipitating antibody in each eluate. These data (column 3, Table IV) show the substantial variation in precipitating capacity of these

TABLE IV

Some Properties of Purified Human Anti-A Antibodies

Antibodies from serum	Antibody fraction	Precipitating antibody / total protein	Minimum concentration for hemagglutination	Complement fixing capacity		Hemolytic capacity (tested on A_1 RBC)
				Amount of antibody used	C′ fixed	
		%	μg N/m	$m\mu g$ N	%	$m\mu g$ N
Serum 1	γG GalNAc eluate	43	0.2	210	51	900*
				300	94	
	γM GalNAc eluate	68	0.7	320	13‡	2000*
				720	12‡	
	γG AR_L0.52 eluate	61	0.04	210	0	1800*
				900	84	
Serum Jos.	γG GalNAc eluate	59	0.3	1060	0	
	γM GalNAc eluate	69	0.001	2200	§	
	γG AR_L0.52 eluate	100	0.01	310	14	
				940	34	
Serum Chris.	γG GalNAc eluate	74	1.0	300	40	>264‖
				400	69	
	γM GalNAc eluate	80	0.01	400	§	18‖
	γG AR_L0.52 eluate	86	0.4	260	58	>274‖
				310	73	
	γM AR_L0.52 eluate	74	0.007	390	46	18‖
				620	81	

* Antibody N giving 40% lysis.

‡ Hog (A + H) BGS was used as antigen.

§ Gave lysis in control tubes with nonsensitized sheep red blood cells.

‖ Antibody N giving 50% lysis.

fractions. γG GalNAc, of serum 1, is only 43% precipitating antibody. γG AR_L0.52 of Jos. was completely precipitated by antigen. The others ranged between these two.

Complement fixation assays on these purified antibodies with soluble hog (A + H) or human MSM as antigens are presented in Figs. 3 *a*, 3 *b*, 3 *c*. Fig. 3 *a* gives results with eluates from serum 1. 94% of the C′ was fixed by 300 mμg of the purified γG antibody from the GalNAc eluate while the γM of

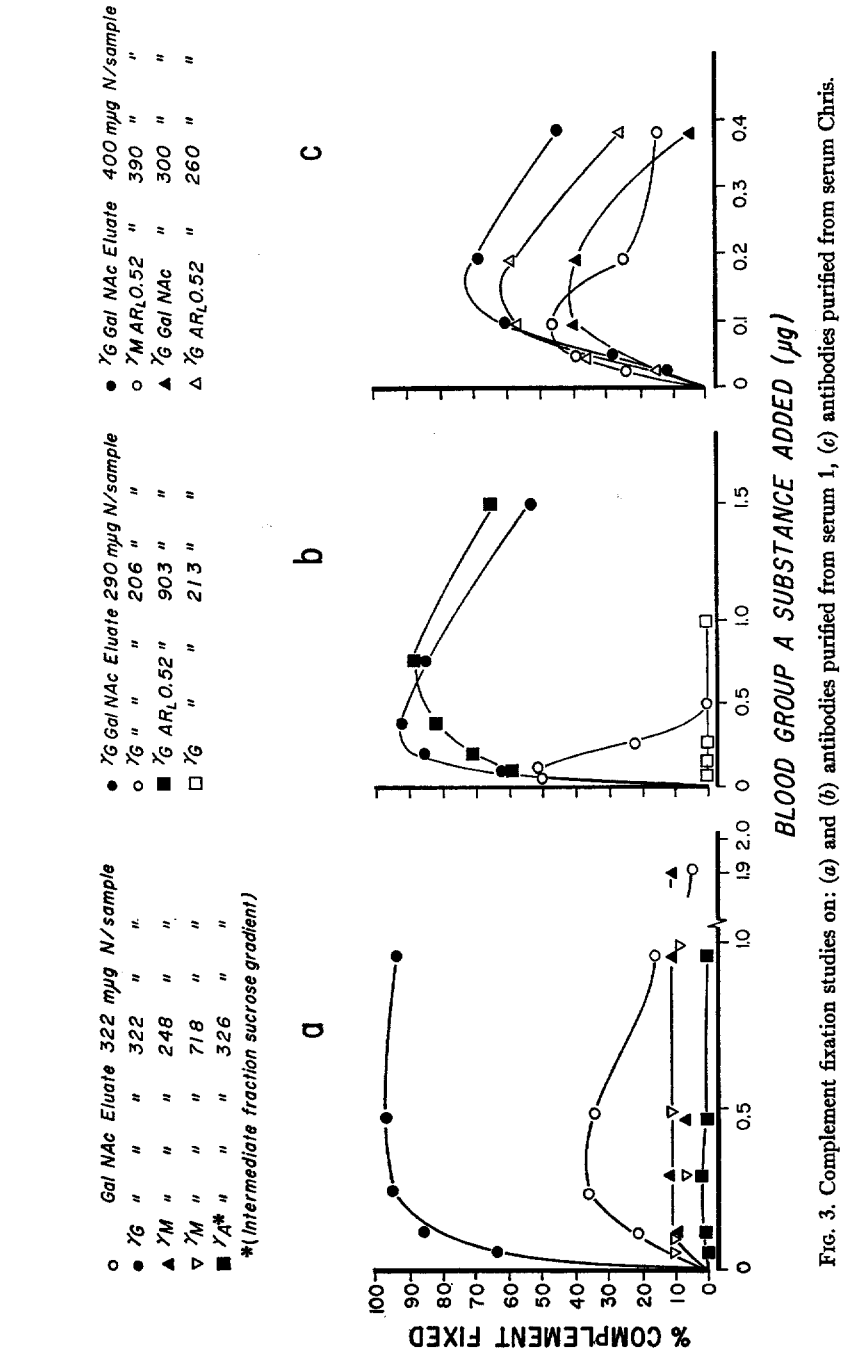

FIG. 3. Complement fixation studies on: (*a*) and (*b*) antibodies purified from serum 1, (*c*) antibodies purified from serum Chris.

the same eluate fixed only 13%, using 284 mμg of antibody N. This latter amount of fixation can hardly be regarded as significant because increasing the quantity of antibody to 718 mμg N did not increase the amount of fixation (cf. Fig. 3 *a*). The substitution of hog A + H blood group substance by human (MSM) as antigen or the use of human instead of guinea pig C′ did not increase the amount of fixation by this antibody. The intermediate fraction of the sucrose gradient obtained from the same GalNAc eluate (see Fig. 1) was rich in γA antibody but it did not fix C′. The γG content of the GalNAc eluate from serum 1 was 46% and with 322 mμg N about 40% fixation was found (Fig. 3 *a*).

Fig. 3 *b* compares the C′ fixation by γG GalNAc and γG AR_L0.52 antibodies from serum 1. The γG GalNAc antibody is approximately three times better in complement fixation than the γG AR_L0.52. 903 mμg N of γG AR_L0.52 were needed to fix approximately to the same extent as 290 mμg N of γG GalNAc antibody. With 206 mμg N, γG GalNAc fixed over 50% while 213 mμg N from γG AR_L0.52 gave no fixation.

Fig. 3 *c* gives the results obtained by C′ fixation using the antibodies purified from Chris. serum, except for the γM GalNAc which always lysed the controls with nonsensitized sheep red cells. The γM antibodies in the AR_L0.52 eluate fix less C′ than any of the γG fractions from the same serum. With γG antibodies the pattern observed is the reverse of that in serum 1; the γG GalNAc antibody is not as good as the γG AR_L0.52. 300 mμg N of γG GalNAc eluate fixed only 40% C′, as compared with the 58% fixed with 260 mμg N for the γG AR_L0.52 eluate. The capacities of different antibodies to fix C′ are summarized in Table IV, columns five and six. Both Jos. and Chris. γM GalNAc lysed sheep red cells and their capacity to fix C′ cannot be estimated. Jos. γG GalNAc did not fix C′ using 1060 mμg N. The γG AR_L0.52 eluate from the same serum gave rather poor fixation (34% with 940 mμg N). Larger amounts of this antibody also lysed sheep red cells.

To test the possibility that the incapacity of γM GalNAc antibody from serum 1 to fix C′ was due to a property of the antigen-antibody complex, rather than a lack of receptor for C′ in the antibody itself, the direct hemolytic assay on A_1 red blood cells was used. The results are given in Fig. 4 *a*. It is clear that the γM GalNAc antibody has, on a weight basis, hemolytic activity comparable with that of the γG AR_L0.52 eluate, but the γG GalNAc eluate is the best of the three. The whole GalNAc eluate was as good as γG GalNAc antibody alone. On a molar basis, however, γM GalNAc had somewhat better hemolytic activity than γG GalNAc. The curves of lysis obtained with γG and γM antibodies from serum 1 had a tendency to stabilize at about 60% hemolysis; for this reason a level of 40% rather than 50% lysis was used as the basis of comparison (see Table IV, last column).

When γG and γM antibodies from serum Chris. were studied in a similar way (Fig. 4 *b*), it was seen that the γM GalNAc and γM AR_L0.52

eluates lysed A_1 human cells and the curves of lysis were absolutely superimposable, but γG GalNAc and γG $AR_L0.52$ eluates failed to lyse the cells with

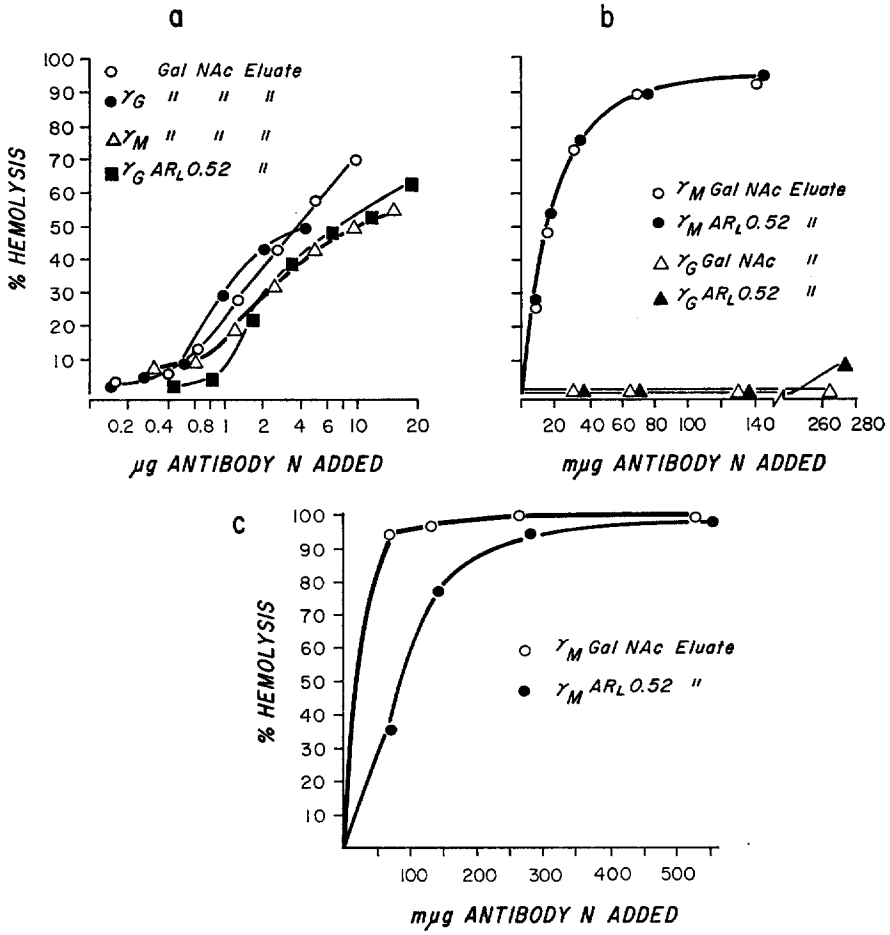

FIG. 4. Hemolytic assay of purified anti-A antibodies: (*a*) Lysis of human A_1 erythrocytes by antibodies from serum 1. $5C'H_{50}$; 0.5 ml, RBC (4.4×10^7 cells/ml); total volume 1.25 ml, incubated 1 hr at 37°C. (*b*) Lysis of human A_1 erythrocytes by purified antibodies from Chris. serum. $10C'H_{50}$; 1.0 ml A_1 RBC (4.6×10^7 cells/ml); total volume of 2.0 ml, incubated 1 hr at 37°C. (*c*) Lysis of sheep red cells by purified γM antibodies from Chris. serum. $2C'H_{50}$; 1.0 ml sheep RBC (2.0×10^5 cells/ml); total volume 2.0 ml, incubated 1 hr at 37°C.

the amount of material used. Fig. 4 *c* shows that although the two γM eluates were equally potent in lysing human A_1 cells, the γM GalNAc eluate was approximately four times better when tested against sheep red cells.

The findings by inhibition of precipitation on the original serum and on

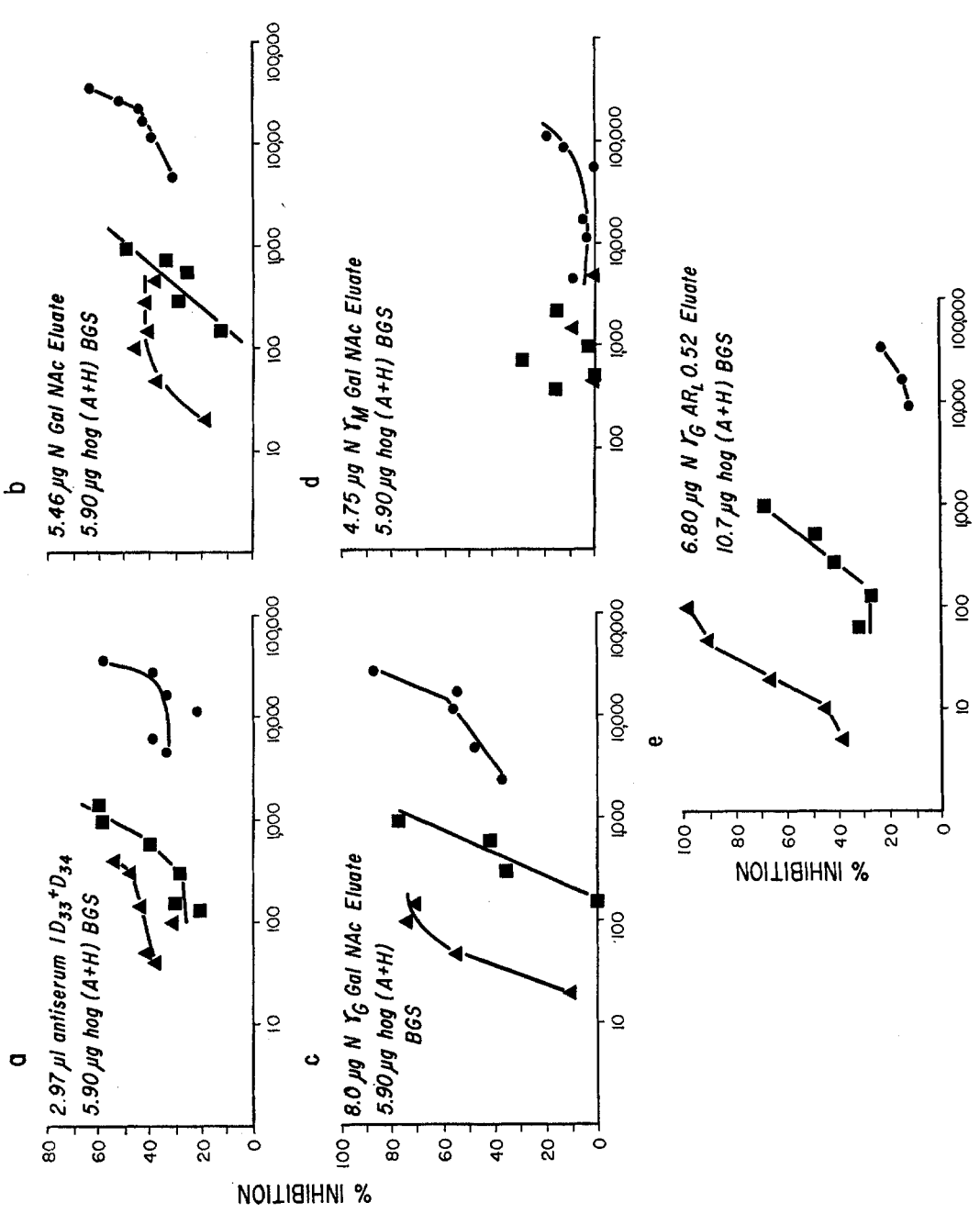

FIG. 5. Inhibition by GalNAc ●, A_3II trisaccharide ■, and AR_L0.52 pentasaccharide ▲ of precipitation of anti-A from serum 1 by blood group A substance.

each eluate are presented in Figs. 5, 6, and 7 in which per cent inhibition is plotted against log inhibitor concentration. All eluates were tested with reduced pentasaccharide $AR_L0.52$, trisaccharide A_5II and with GalNAc. Fig. 5 *a* shows the inhibition curves of antiserum 1. The curves are nonlinear with a smooth slope indicating heterogeneity; this is particularly evident with pentasaccharide $AR_L0.52$. Increasing capacity to inhibit precipitation is seen in going from the mono-, to the tri-, to the pentasaccharide. In Fig. 5 *b*, inhibition assays on the GalNAc eluate of serum 1 show that the curve with $AR_L0.52$ reaches a plateau at 50% inhibition. In Fig. 5 *d*, the data using the γM GalNAc eluate from serum 1 are given. Significant inhibition was not obtained using as much as 4,920 mμM of $AR_L0.52$, 2,400 mμM A_5II; 107,000 mμM GalNAc gave only 20% inhibition. The limited quantities of $AR_L0.52$ and A_5II did not permit the use of larger amounts. The γG GalNAc eluate of the same serum gave inhibition curves with steep slopes, the $AR_L0.52$ being almost parallel to the A_5II curve (Fig. 5 *c*). Approximately 10 times more trisaccharide A_5II was necessary to reach equivalent inhibition and 11 times more GalNAc than A_5II were needed for 50% inhibition. In Fig. 5 *e*, the γG $AR_L0.52$ eluate of serum 1 shows that good inhibition was reached with both $AR_L0.52$ and A_5II, but extremely poor inhibition was seen with GalNAc.

Fig. 6 gives the results for Jos. antiserum and its eluates. Jos. antiserum was not like serum 1 in that the curves of inhibition with A_5II and $AR_L0.52$ are very steep (Fig. 6 *a*). In Fig. 6 *b*, the γG GalNAc eluate of Jos. gave inhibition curves quite similar to those for the γG GalNAc eluate of serum 1 (Fig. 5 *c*), although higher concentrations of inhibitor (120 and 31,000 mμM of $AR_L0.52$ and GalNAc respectively were required for 50% inhibition). The similarity between this eluate (Fig. 6 *b*) and the Jos. antiserum (Fig. 6 *a*) is striking. With the γM GalNAc of Jos. very few points could be set up, owing to the scarcity of the oligosaccharides and the very high levels of inhibitors needed. Significant inhibition with all three inhibitors was obtained; 800, 3,000, and 37,000 mμM gave 50% inhibition with $AR_L0.52$, A_5II, and GalNAc in that order; these figures must be only considered as rough estimates. The γG $AR_L0.52$ eluate of Jos. was inhibited only by $AR_L0.52$ at a concentration comparable to the γG GalNAc eluate; 190 mμM gave 50% inhibition. No substantial inhibition was observed with A_5II and GalNAc, although very high levels of inhibitor were used.

Antiserum Chris. (Fig. 7 *a*) resembled antiserum 1 in its inhibition curves, especially that with GalNAc. The γG GalNAc fraction (Fig. 7 *b*) differed from those of serum 1 and of Jos. in that $AR_L0.52$ was not any better as an inhibitor than A_5II. The γG $AR_L0.52$ eluate (Fig. 7 *c*) was not inhibited by GalNAc and the inhibition by A_5II and $AR_L0.52$ was like that seen with whole serum (Fig. 7 *a*) except that the curves were steeper. Antiserum Chris. had γM antibodies in both the GalNAc and $AR_L0.52$ eluates. Inhibition assays are

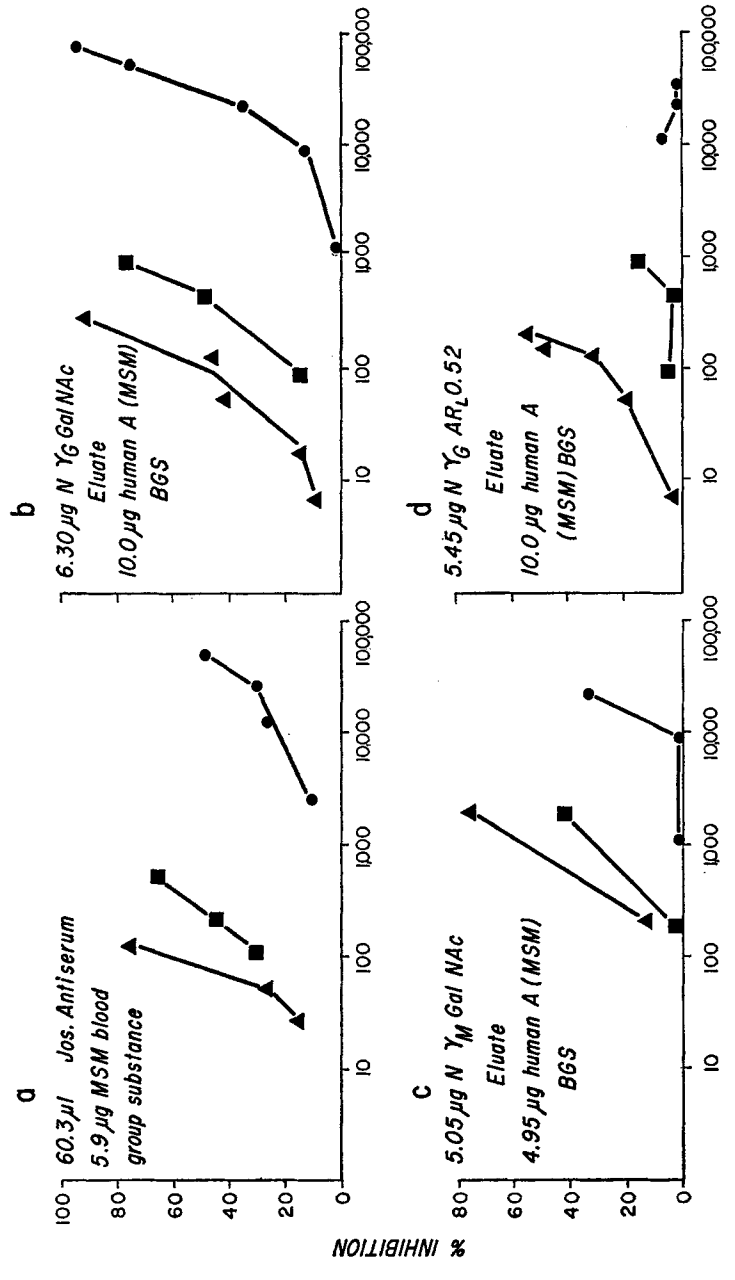

FIG. 6. Inhibition by GalNAc ●, A_5II trisaccharide ■, and $AR_L0.52$ pentasaccharide ▲ of precipitation of antibodies from Jos. serum by blood group A substance.

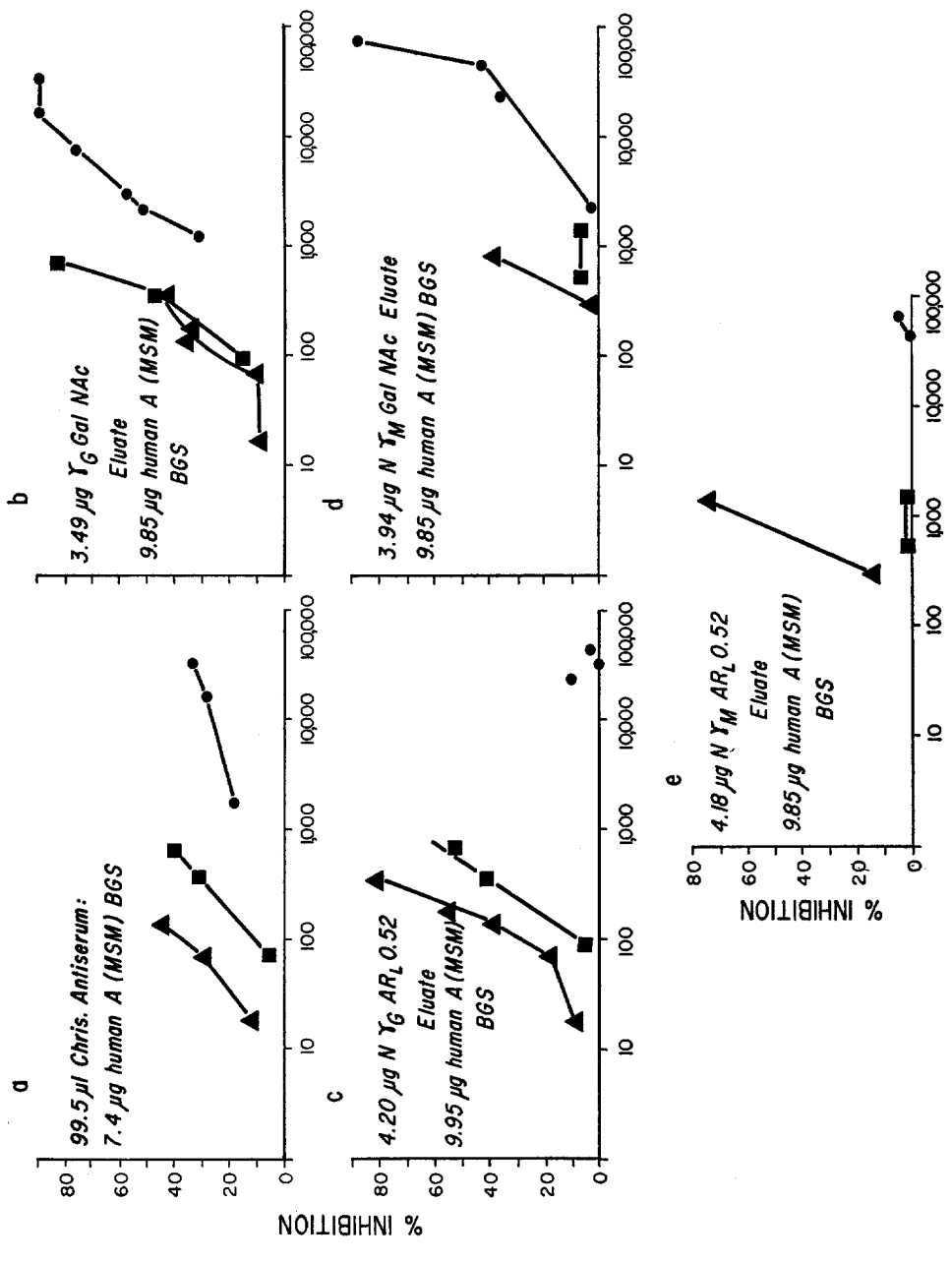

FIG. 7. Inhibition by GalNAc ●, A_3II trisaccharide ■, and AR_L0.52 pentasaccharide ▲ of precipitation of antibodies from Chris. serum by blood group A substance.

given in Figs. 7 *d* and *e* respectively. They resemble each other in several respects. (*a*) $AR_L0.52$ is equally good as an inhibitor with both; (*b*) A_1II does not inhibit at all in the range tested; (*c*) With GalNAc, the GalNAc eluate gave 50% inhibition with 50,000 mμM, while at this level the $AR_L0.52$ eluate gave none.

The studies of the antibodies by inhibition of precipitation are summarized in Table V. The figures correspond to the amount of oligosaccharide giving 50% inhibition of precipitation.

TABLE V

Concentration of Oligosaccharides Giving 50 Per Cent Inhibition of Precipitation of Anti-A Fractions by Human and Hog A Substance as Compared with K_0 by Equilibrium Dialysis

Serum	Antibody fraction	Inhibitor (mμmoles)			K_0
		$AR_L0.52$	A_1II	GalNAc	
No. 1	γG GalNAc	52	530	6000	8×10^4
	γM GalNAc	>5000	>2400	>107000*	
	γG $AR_L0.52$	12	430	>34000	1×10^5
Jos.	γG GalNAc	120	450	31000	2×10^3
	γM GalNAc	800	3000	37000	
	γG $AR_L0.52$	190	>900	>35000	2×10^4
Chris.	γG GalNAc	360	360	2200	2×10^4
	γM GalNAc	1200	>1400	50000	
	γG $AR_L0.52$	180	580	>44000	5×10^4
	γM $AR_L0.52$	750	>1400	>65000	

* By extrapolation beyond the last point measured to 50%, a value of about 300,000 would be obtained.

Studies by equilibrium dialysis on the purified antibodies are given in Figs. 8, 9, and 10, corresponding to sera 1, Jos., and Chris. respectively. In each one of these the Scatchard (*a*) and Sips (*b*) plots are presented. The binding constants calculated from the Sips plot and the heterogeneity index "*a*" are given in each figure.

γG GalNAc and γG $AR_L0.52$ eluates from serum 1 (Fig. 8) show binding constants of 8×10^4 and 1×10^5 liters/mole erspectively; values well within experimental error. The γG $AR_L0.52$ antibody was less heterogeneous than γG GalNAc. In the Scatchard plot, the values of r can be extrapolated to 2. The γM GalNAc eluate of this serum was also studied, but no binding was obtained with it, indicating a very low constant of binding.

Figure 9, gives the data on the γG eluates from Jos. serum. The binding

constants, 2×10^3 and 2×10^4 liters/mole for the γG GalNAc and γG $AR_L0.52$ were substantially lower than those obtained with the antibodies from serum 1. Both had 0.6 for the heterogeneity index. The low value of the binding constants together with the limited amount of antibody did not permit obtaining r values over one and extrapolation of the γG GalNAc eluate was not possible.

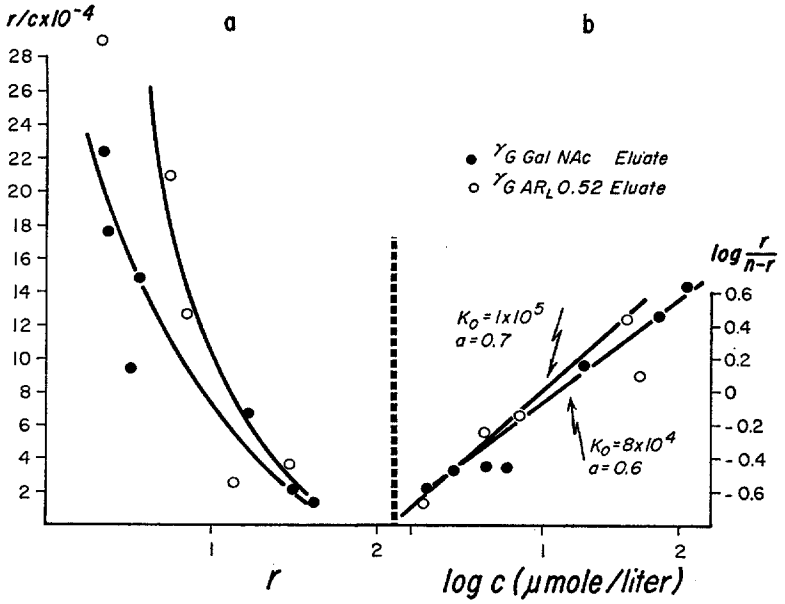

FIG. 8. Scatchard (*a*) and Sips (*b*) plots of equilibrium dialysis experiments with γG antibodies from serum 1 and ^3H–$AR_L0.52$ as hapten.

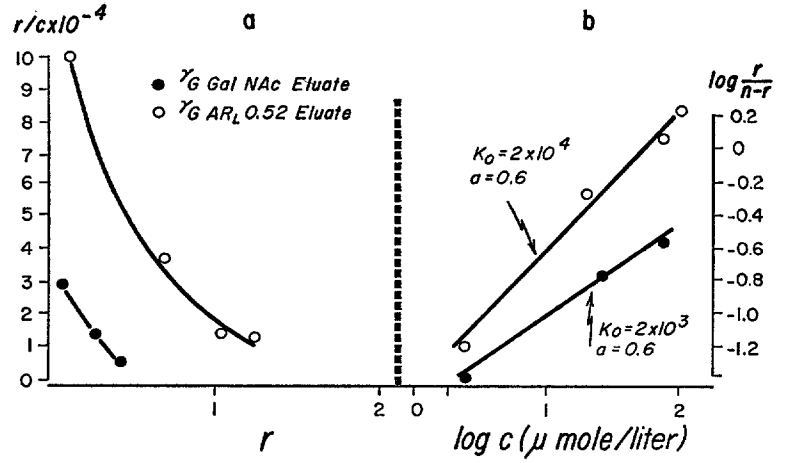

FIG. 9. Scatchard (*a*) and Sips (*b*) plots of equilibrium dialysis experiments with γG antibodies from Jos. serum and ^3H–$AR_L0.52$ as hapten.

Chris. γG $AR_L0.52$ eluate (Fig. 10) had a constant K_0 of 5×10^4, somewhat higher than that of the γG GalNAc from the same serum ($K_0 = 2 \times 10^4$); both of these antibodies had an a value equal to 0.6.

The light chains in these purified eluates were assayed by immunodiffusion against anti-κ and anti-λ antisera. The results can be summarized as follows: serum 1 γG GalNAc: mostly κ determinants; serum 1 γM GalNAc: mostly λ determinants; serum 1 γG $AR_L0.52$: κ and λ.

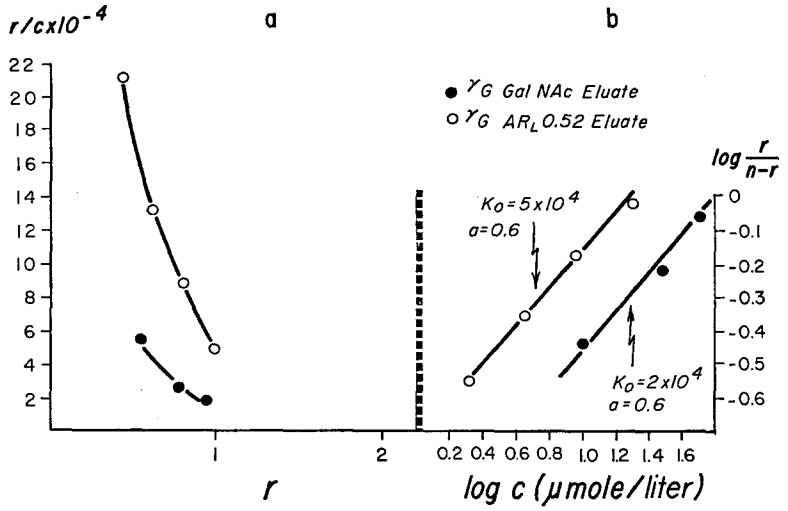

FIG. 10. Scatchard (a) and Sips (b) plots of equilibrium dialysis experiments with γG antibodies from Chris. serum and ^3H-$AR_L0.52$ as hapten.

All the eluates from Chris. and Jos. antisera contained κ and λ determinants in substantial amounts. The anti-A antibodies even from purified eluates thus seem to be still heterogeneous in this respect.

DISCUSSION

The procedure used to purify the antibodies from anti-A sera was designed to maximize the difference between the GalNAc and $AR_L0.52$ eluates, especially with respect to the properties of the combining site. This was done by repeating the extraction with GalNAc until essentially all the antibody capable of being eluted by this sugar was taken out. Only then was elution with $AR_L0.52$ attempted. Several properties of the antibodies and especially the findings with inhibition by oligosaccharides, must be interpreted on this basis.

The GalNAc eluates always contained more antibody than did the $AR_L0.52$ eluates and the former were invariably more heterogeneous with respect to the classes of immunoglobulins present (Tables II and III). All of the GalNAc eluates contained γM and γG antibodies but only one $AR_L0.52$ eluate (Chris.)

had any γM antibodies. It is not possible to decide whether the other two individuals whose sera had no γM in their $AR_L0.52$ eluates had failed to make a γM antibody of sufficient binding affinity to resist elution by GalNAc and yet be eluted by $AR_L0.52$, or whether they made γM antibody of higher affinity than Chris., such that it was not eluted by the limited amounts of $AR_L0.52$ used and remained on the column. Since γM antibodies have a higher valence than γG antibodies, a given concentration of eluant would tend to elute the latter more rapidly than the former, assuming binding sites of the same affinity.

The total recovery from the column (Table II) was 72% in the best case, and it was not possible to elute additional antibody with KI or KCNS (35, 36). The recoveries of antibody are comparable to those obtained with antidextran eluted from Sephadex columns by isomaltose oligosaccharides (37–39).

The data on the purified antibody fractions (Table IV) show that there is no correlation between the hemagglutinating capacity of a given antibody for A erythrocytes and whether it is γG or γM anti-A. With serum 1, more antibody N was required to give detectable hemagglutination with the γM GalNAc eluate than with the γG GalNAc eluate, as had been found earlier for two other anti-A sera (5), while with Jos. and Chris. the reverse was the case. Other workers (4, 40–42) generally report γM antibodies to be more potent in agglutination than γG antibodies per unit weight, but in other instances, the reverse has been found.[6] The current findings with anti-A indicate that both types of behavior may occur within a single antigen-antibody system.

It is especially significant, however, that with all three sera the hemagglutinating potency of the γG GalNAc antibody was always less than that of the γG $AR_L0.52$ eluate, a result in accord with the hypothesis that the latter is eluting antibody with larger size combining sites and hence with higher affinity.

Although the purified antibody solutions varied in precipitability by soluble blood group substance from 43–100%, it is not believed that the lower values represent impurities, and all analyses are based on the total protein N of the solutions. On the micro scale used, solubility of antigen-antibody precipitates is appreciable (27) and this would tend to give low results. Moreover, serum proteins other than γM, γG, and γA immunoglobulins would not be detected in immunoelectrophoresis. By agar diffusion traces of serum albumin could sometimes be seen. In addition, assays by radial diffusion showed the γM, γG, and γA in the GalNAc or $AR_L0.52$ eluates to account for 100% of the protein. Finally, equilibrium dialysis data on the γG GalNAc eluate of serum 1 which showed the lowest amount of precipitable antibody gave an extrapolated valence of 2, based on total N content indicating the absence of nonantibody protein.

[6] Schulkind, M., M. Herzberg, and J. A. Robbins. 1968. Antibacterial antibodies. II. Specific secondary biological properties of rabbit antibodies to *Brucella abortus* and *Bordetella pertussis*. Submitted for publication.

With respect to C′ fixing capacity, variations were also found even with the same class of antibody from a given individual. The γG GalNAc eluate from serum 1 was more potent in fixing C′ than the γG $AR_L0.52$ eluate, but the reverse was the case with Jos. and Chris. The basis for this variation is not clear, but could in part be related to differences in the γG subclasses, since anti-A antibodies generally tend to be mixtures of various subclasses of γG (43) and Ishizaka et al. (44) found differences in C′ fixing potency of aggregated human myeloma globulins of different γG subclasses. With human antidextran, all of which was of the γG2 subclass (43), the antibody eluted by larger oligosaccharides was more potent in C′ fixation than that eluted with smaller oligosaccharides (38).

An unusual specificity difference was also noted between the γM GalNAc and γM $AR_L0.52$ eluates of Chris. While both were equally potent in lysis of human A_1 erythrocytes, the former was four times better in lysing sheep erythrocytes. This difference may provide another parameter of heterogeneity of γM antibodies and could also throw light on the nature of the human A–sheep erythrocyte Forssman antigen system (45, 46). The capacities of the antibody fractions to fix C′ with soluble blood group substances and to hemolyze human A_1 cells in the presence of complement did not correlate. In hemolysis, the γM eluates were always better than the γG eluates on a molar basis, whether or not they were poorer in C′ fixation. The hemolysis of an erythrocyte generally requires a single molecule of γM antibody to fix C′1a on to the surface of an erythrocyte, while it is thought that two γG antibody molecules must be at the same site for hemolysis to occur (12). The differences between γM and γG noted in this study for serum 1 are relatively small, while with Chris. they are very great. Thus, the major factor involved in variations in hemolysis as in C′ fixation is probably ascribable largely to antibody heterogeneity in the γM, as well as in the γG fractions. Plotz et al. (47) have described a mouse γM antibody unable to fix C′, and Hoyer et al. (48) found that only 21% of a rabbit γM antiazobenzenearsonate antibody was capable of fixing C′1a.

Ishizaka et al. (49), studying rabbit anti-A, found that γG was better than γM when tested by C′ fixation using soluble antigen, but the reverse was true when the test was done with red blood cells or insoluble PLA blood group substance as antigen, indicating that the capacity of a given antibody to fix C′ is a property that can depend on the physical state of the antigen, as is also supported by our data using hemolysis and C′ fixation.

A most important finding is the fractionation of anti-A antibodies with respect to the sizes of their combining sites as measured by differences in their relative capacity to be inhibited by small as compared with large oligosaccharides. It was to be expected that after the prior extensive elution with GalNAc, the $AR_L0.52$ eluates would be relatively poorly, if at all, inhibited by GalNAc, and this turned out to be so in all instances. The $AR_L0.52$ eluates were only inhibited by $AR_L0.52$, and in some instances, by the trisaccharide A_6II.

With serum Chris. this was true for the γM eluates as well as the γG eluates. These data do not imply that GalNAc does not inhibit but only that it is at a very great competitive disadvantage in displacing the A determinants on the polyvalent A macromolecule. The findings with the γM eluates of Chris. show that γM sites may be complementary to sizes larger than a monosaccharide and thus may not differ significantly in the sizes of their determinants from those of γG antibodies.

All γM anti-A fractions studied required larger concentrations for inhibition than did the corresponding γG antibodies from the same individual. With the eluates from Jos., the concentrations of all three oligosaccharides needed for 50% inhibition were one order of magnitude greater than with the γG eluates. In studies in other systems, Jaton et al. (50) reported that precipitation by antigen of γM and γG anti-uridine antibodies prepared to artificial uridine-containing proteins was equally well inhibited by uridine, but Groff et al. (51) with phenylarsonate antibodies found about 10-fold higher concentrations of phenylarsonate analogues were needed to give comparable inhibition with γM than with γG antibodies.

With all γG eluates, except the Jos. γG $AR_L0.52$, the inhibition by A_5II was relatively constant, the amount needed for 50% inhibition ranging from 360 to 580 mμM, while in simultaneous assays the corresponding values for $AR_L0.52$ varied from 12 to 360 mμM. A_5II differs from $AR_L0.52$ in two important respects: (*a*) it lacks the fucose substitution on the subterminal galactose; (*b*) it is a type 1 determinant (52) being linked β-D-Gal-(1 $\rightarrow$ 3)-D-GNAc while the $AR_L0.52$, a type II determinant is β-D-Gal-(1 $\rightarrow$ 4)-D-GNAc. Whether the uniformity of the inhibition with A_5II represents a uniform contribution of its three sugars to the total energy of binding or is due to chance is not known.

Another major finding is that, for each individual, the binding constants of the $AR_L0.52$ eluates were higher than those for the GalNAc eluates, although the values with serum 1 are very close. This parallels the findings with the inhibition data and is what one would expect on the hypothesis that the $AR_L0.52$ antibodies had larger size combining sites.

The values of the association constants, with one exception, 2–10 $\times$ 10^4 are generally on the low side of those reported for many systems but are only slightly lower than the values found by Karush (53) of 1 and 2 $\times$ 10^5 for anti-Lac antibodies and of the same order as those 8–20 $\times$ 10^4 found for rabbit and horse type VIII antipneumococcal antibodies by Pappenheimer et al. (54). Jaton et al. (50) reported 5.6 $\times$ 10^4 for the association constant of anti-uridine antibodies. The Jos. γG GalNAc eluate gave an extraordinarily low value of 2 $\times$ 10^3. The heterogeneity index in the Sips plots were still very low indicating substantial heterogeneity in the eluates. These values and those of Pappenheimer et al. (54) and Merler et al. (55) represent the first measurements with antigenic determinants of naturally occurring carbohydrates.

Values for binding constants of anti-A and anti-B based on measurements of direct binding of γM and γG antibody to erythrocytes have also been published (56–58). These values tend to be much higher than those reported here, but since they are based on heterogeneous systems and both the antigen and antibody are multivalent, they are not measuring the same property.

SUMMARY

Human antibodies to blood group A substance were purified by absorption on columns of insoluble polyleucyl hog blood group A + H substance and eluted first with *N*-acetylgalactosamine and then with an A active reduced pentasaccharide $AR_L0.52$. The γM and γG antibodies in these eluates were separated by density gradient centrifugation. The antibodies were studied for their relative capacities to be inhibited by various blood group A active oligosaccharides. Antibodies eluted by the *N*-acetylgalactosamine could be inhibited by *N*-acetylgalactosamine, as well as by lower concentrations of A active tri- and pentasaccharides, while those eluted by the pentasaccharide $AR_L0.52$ could only be inhibited by the two oligosaccharides, but not by *N*-acetylgalactosamine, indicating that the *N*-acetylgalactosamine eluate had more antibodies with smaller size combining sites than the $AR_L0.52$ eluate. Measurements by equilibrium dialysis gave values ranging from 2×10^3 to 1×10^5 M^{-1} and the values obtained with the $AR_L0.52$ eluate were somewhat higher than those with the GalNAc eluate. Only one of three anti-A sera had γM anti-A in the $AR_L0.52$ eluate, while all three had γM in the *N*-acetylgalactosamine eluate. Data on the precipitating, hemagglutinating, complement fixing, hemolytic properties of the eluted antibodies, and of their content of κ and λ light chains are given.

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