HETEROGENEITY OF RABBIT IgM ANTIBODY AS DETECTED BY C'1a FIXATION*

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Macroglobulin (IgM) antibodies efficiently induce complement-dependent erythrocyte lysis (1–3). By the use of the C'1a fixation and transfer (C'1aFT) test, and by direct lysis, Borsos and Rapp have demonstrated that a single molecule of rabbit 19S (IgM) anti-Forssman hemolysin is sufficient to sensitize a sheep erythrocyte to the lytic action of guinea pig complement (4). The C'1aFT test measures the number of C'1a molecules fixed at cell surfaces by antigen-antibody complexes. C'1a fixation by each 19S IgM antibody molecule has been reported for human IgM anti-A isoagglutinin (5) as well as rabbit anti-Forssman (6) antibody. The amount of Forssman antibody was determined by quantitative precipitin analysis using the water soluble antigen (6) and the amount of human IgM anti-A antibody was determined by measuring the adsorption of radio-labeled antibody to erythrocytes (5).

We have obtained additional quantitative information about complement fixation using specifically purified antibody. Rabbit IgM anti-benzenearsonate (anti-R) antibody was purified by means of a hapten-cellulose immunoadsorbent (7) and assayed by the C'1aFT test. While previous studies (5, 6) suggested that all IgM antibody molecules could fix C'1a, only a fraction of the anti-R IgM antibody molecules fixed C'1a. The IgM antibody molecules capable of fixing C'1a were more negatively charged at pH 8.6 than the bulk of the IgM anti-R antibody.

Materials and Methods

Antisera.—Young adult New Zealand albino rabbits were immunized with edestin azobenzenearsonic acid (R-Ed) (7) by two different schedules which had been found to stimulate IgM antibody response to this antigen. Animals immunized according to schedule A received 7–10 mg of R-Ed intravenously three times a week for 2 wk with supplementary injections

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of 5 μg endotoxin (*Salmonella enteritidis* somatic polysaccharide) intravenously with the first injection of each week. The animals were bled on the 5th and 6th days after the last injection. In schedule B, the animals received R-Ed coated on acrylic particles (AB Bofors, Nobelkrut, Sweden) by the method of Torrigiani and Roitt (8). The 11% suspension of 0.8 μ particles used for immunization had a protein content of 0.7–1.2 mg/ml. The animals received intravenous injections of 3 ml of this suspension on the first day and 2 ml 4 and 9 days later. They were bled on the 5th and 6th days after the last injection. The sera from each rabbit were pooled and stored at −20°C until used.

Antigens.—The immunizing (R-Ed) and test (R-BSA)[1] antigens were prepared by coupling diazotized *p*-arsanilic acid to the protein carriers. Details of preparation and characterization have been reported (7). The R-BSA used for the free boundary electrophoresis experiments had 33 arsenic atoms per molecule of BSA and the R-BSA used for quantitative precipitin analyses had 39 arsenic atoms per molecule of BSA.

DEAE-Cellulose Chromatography.—Globulin fractions of some antisera were obtained by ammonium sulfate precipitation and then depleted of IgG by DEAE-cellulose chromatography. Proteins which precipitated in 50% saturated ammonium sulfate at room temperature, pH 7.9, were dissolved in saline and dialyzed against 0.035 M phosphate buffer, pH 7.5. Globulin prepared from 60 ml of serum was applied to a 2.3 × 100 cm column of DEAE-cellulose previously equilibrated with the same buffer at room temperature. After thorough washing of the column with this buffer, the adsorbed protein was eluted with 0.4 M phosphate, pH 4.35. This globulin fraction did not contain any IgG when examined by immunoelectrophoresis.

Preparation of Purified Antibody.—Preparation and use of the benzenearsonic acid–cellulose immunoadsorbent has been previously described (7). Serum and IgG-depleted globulin fractions were dialyzed against 10% borate saline buffer, pH 7.85, and applied to columns of the immunoadsorbent. Antibodies were eluted with 1 M NaCl, 0.05 M in phosphate, pH 3.0, followed by 0.1 N HCl.

Antibody fractions prepared from the sera of individual rabbits were neutralized, concentrated by negative pressure ultrafiltration, and dialyzed against 10% borate saline buffer, pH 7.85. IgG and IgM antibodies were then separated by gel filtration through Sephadex G-200 columns equilibrated with the same buffer (9). Samples of 10 to 135 mg in 2–5 ml were applied to a 1 × 120 cm column run at room temperature with a flow rate of 4 ml/hr.

DEAE-cellulose chromatography removed most of the IgG from antibody prepared from large pools of antiserum. After dialysis against 0.04 M phosphate, pH 8.0, the antibody (0.5–1.2 g) was applied to DEAE-cellulose columns (2.3 × 35 cm) equilibrated with the same buffer and washed until the effluent contained no significant protein. The IgM antibodies were eluted with 0.4 M phosphate, pH 8.0, and further purified by gel filtration twice through Sephadex G-200.

Antibody fractions prepared from IgG-depleted globulin were dialyzed against 10% borate saline buffer and used as dilute (0.04–0.08 mg/ml) solution.

Characterization of the Antibody.—The IgM anti-benzenearsonate antibody prepared from pooled antisera was characterized by ultracentrifuge studies using a Beckman Spinco model E analytical ultracentrifuge (7), immunoelectrophoresis (10), free boundary electrophoresis in the presence of antigen (7), and quantitative precipitin assay (12). Acrylamide disc gel electrophoresis was done using the method of Reisfeld and Small (11) except that urea was not added.

C′1a Fixation and Transfer Test.—The number of C′1a molecules fixed at the cell surface by antibody-antigen complexes was measured by the C′1a FT test (4) with a single modification: volumes of 0.1 ml of antibody dilution, erythrocytes, and C′1a were used in the first or fixation stage.

[1] BSA, bovine serum albumin.

Preparation of Benzenearsonic Acid–Azo-Sheep Erythrocytes (R-SRBC).—Erythrocytes from a single sheep bleeding were used after washing and incubation with EDTA (4). Ingraham's method (13) was modified to provide somewhat more effective hapten-coupled erythrocytes for these experiments by increasing the hapten/cell ratio. All reagents were kept in an ice bath. 1 mmole (217 mg) of *p*-arsanilic acid was dissolved in 1 ml of distilled water and 1 ml of 1 N sodium hydroxide. 1 ml 4 N hydrochloric acid was added to 1.1 mmole sodium nitrite (76 mg) in 2 ml distilled water, and this mixture added to the arsanilic acid and allowed to react for 15 min. 1 ml of 0.2 M urea was added and the solution brought to pH 7.4 with 1 N sodium hydroxide. The diazotized arsanilic acid was slowly added to 20 ml of a 10% (v/v) suspension of sheep erythrocytes in 0.15 M sodium chloride buffered with 0.01 M phosphate at pH 7.4 The coupling was allowed to proceed for 25 min and the cells were washed three times with Veronal-buffered saline (4, 14). If not used immediately, the cells were stored at 4°C in 0.075 M sodium chloride solution buffered at pH 7.4 with 0.075 M phosphate. Before use, the cells were carefully washed three times with Veronal-buffered saline to remove phosphate (15).

Protein Measurement.—The rabbit IgM protein standards were determined by sulfuric acid digestion and nesslerization (7). These were used to calibrate the Lowry phenol method (16) for protein samples of 5–40 μg and to determine the extinction coefficient of the purified rabbit macroglobulin antibody. The extinction coefficient in 10% borate buffer, pH 7.85, was 13.2 ± 0.2 ($E^{1\%}_{280\ m\mu}$) using a Zeiss spectrophotometer. This macroglobulin antibody was assumed to contain 14.5% nitrogen (17).

Preparative Electrophoresis.—A Brinkmann Elphor preparative electrophoresis apparatus was operated at 2500 v and 100 ma for 180 min at 5°C (6). The buffer in the separation chamber was 0.03 M Tris HCl, pH 8.6. The sample was injected at 2 ml/hr, and the buffer flow in the separation chamber was 100–200 ml/hr. 48 fractions were collected.

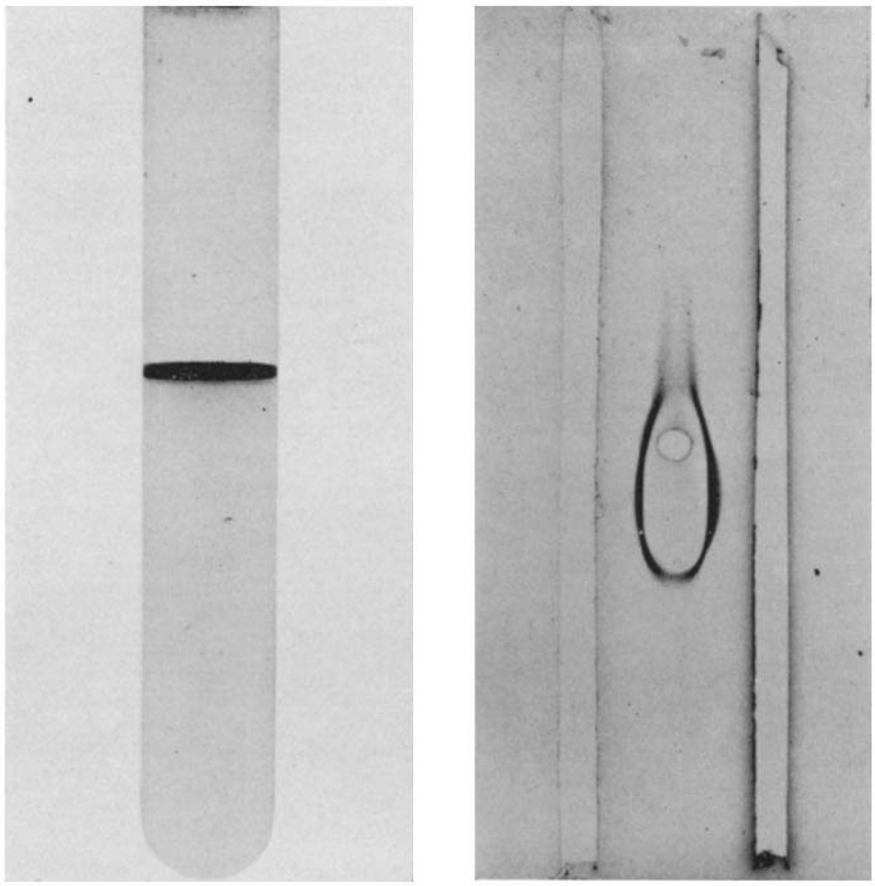
Iodination of Proteins.—A slight modification of McFarlane's method was used to trace-label proteins with ^{131}I (18, 19). The labeled proteins had 0.1–1.1 atoms of ^{131}I per protein molecule and specific activities of 1.0–2.5 μc/mg. Over 99% of the radioactivity of labeled purified antibody preparations was precipitated when a small sample was mixed with an equal volume of 20% trichloroacetic acid. The labeled globulin preparations were 90–95% precipitable under these conditions. Radioactivity was measured using a Nuclear-Chicago (Des Plaines, Ill.) crystal scintillation detector and at least 5000 counts were measured for each 2.5 ml sample.

Buffer Solutions.—Composition of the borate buffer, pH 8.4, and the 10% borate saline, pH 7.85, are given in reference 7. The Veronal-buffered saline (VB-saline) and Veronal-buffered saline-sucrose (VB–saline-sucrose) of low ionic strength used in the C′1aFT test were prepared as described in references 4 and 20. The Tris-HCl buffers for the preparative electrophoresis were prepared by adjusting a 0.09 M Tris [tris (hydroxymethyl) aminomethane] solution to pH 8.6 with hydrochloric acid to obtain the 0.09 M buffer and then diluting with distilled water to obtain the 0.03 M buffer.

EXPERIMENTAL RESULTS

The IgM antibody preparations were purified from individual sera or pools of antisera produced by rabbits during a short intensive immunization. Acrylamide disc gel electrophoresis of the purified IgM demonstrated a single band at the gel interface with barely detectable traces of faster moving components (Fig. 1 *a*). As the material does not easily penetrate the gel, the single heavy band does not imply homogeneity of the macroglobulin but does indicate that the preparation is essentially free of lower molecular weight material. Immuno-

electrophoretic analyses were consistent with the presence of a single IgM component (Fig. 1 *b*). Ultracentrifuge studies revealed a large symmetrical peak with a sedimentation coefficient ($s_{20,w}$) of 18.6S at a concentration of 5.45 mg/ml (Fig. 2). A faster moving component (approximately 30S) representing less than 8% of the protein was present in samples stored at 4°C in borate saline. The concentration estimate was based on area analysis with radial dilution corrections.



(*a*) (*b*)

FIG. 1. (*a*) Acrylamide gel disc electrophoresis of purified rabbit IgM anti-benzenearsonate antibody (0.3 mg). The anode is towards the bottom of the figure. (*b*) Immunoelectrophoresis of purified rabbit IgM anti-benzenearsonate antibody (5.45 mg/ml). The antiserum in the left trough is sheep anti-rabbit IgM. The antiserum in the right trough is sheep anti-whole rabbit serum. The anode is towards the top of the figure. The well and troughs were each filled once.

All the protein in an IgM antibody preparation (pool 1, see Table I) formed soluble complexes when excess antigen (R-BSA) was added and the sample examined by free boundary electrophoresis (7). Control experiments demonstrated that 5% contamination with nonantibody protein would have been detected by this method. The IgM antibody was 77% precipitable using R-BSA as antigen in microquantitative precipitin determinations (12).

Ouchterlony analyses demonstrated no reaction between the purified IgM antibody and goat antiserum against rabbit Fc-fragment or goat antiserum against rabbit colostral IgA absorbed with IgG (b4/b5 heterozygote) to remove

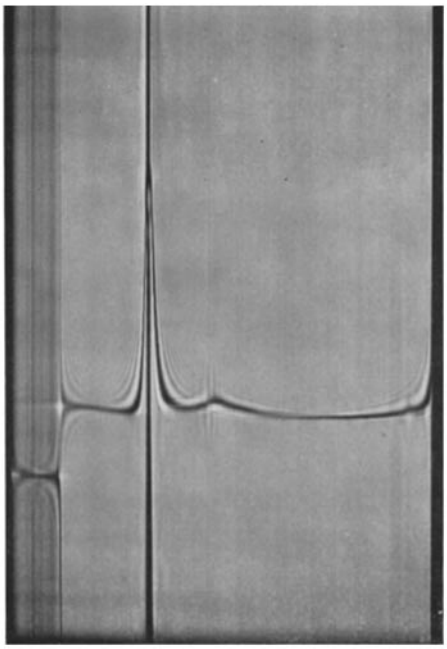


FIG. 2. Analytical ultracentrifuge pattern of purified rabbit IgM anti-benzenearsonate antibody (5.45 mg/ml) in borate saline buffer pH 7.85 at 20°C. Photograph taken 16 min after reaching speed (59,780 rpm).

anti-light chain antibodies. As both goat sera gave good reactions with their homologous antigens, there was little if any polymerized IgG or IgA in the IgM preparation.

The above data indicate that the preparations of specifically purified IgM anti-benzenearsonic acid antibody were at least 95% specific antibody and essentially free of other serum protein contaminants.

Results of the C'1a Fixation and Transfer Test.—The number of C'1a molecules fixed by IgM antibody was determined for eight antibody samples ob-

tained from individual rabbits and two preparations obtained from rabbit serum pools. Benzenearsonic acid–azo-sheep erythrocytes (R-SRBC) were used as the antigen in the first stage of the C'1aFT test. Table I gives the results obtained using antibody eluted from the immunoadsorbent with the pH 3 phosphate–sodium chloride buffer. All the protein in the eluates was assumed to be antibody for these calculations. The uncorrected ratios of C'1a molecules fixed to the number of antibody molecules were between 0.09 and 0.16. This represented a marked difference from the expected ratio of 1.0 (5, 6).

TABLE I

*C'1a Fixation by IgM Anti-Benzenearsonate Antibody**

Source of purified IgM antibody	Immunization schedule	Antibody concentration‡	Antibody molecules/ml§	C'1a molecules fixed/ml‖	C'1a molecules fixed / Antibody molecules
		mg/ml			
Pool 1 (32 rabbits)	A	5.45	36.5×10^{14}	5.0×10^{14}	0.14
^{131}I-labeled pool 1	A	0.66	4.4×10^{14}	6.8×10^{13}	0.16
Pool 2 (6 rabbits)	B	0.16	1.1×10^{14}	1.5×10^{13}	0.14
Rabbit V407	B	0.08	5.4×10^{13}	8.7×10^{12}	0.16
Rabbit V404	B	0.11	7.3×10^{13}	1.1×10^{13}	0.15
Rabbit C28	A	0.21	1.4×10^{14}	1.3×10^{13}	0.10
Rabbit C29	A	0.20	1.3×10^{14}	1.1×10^{13}	0.09
Rabbit C30	A	0.29	1.9×10^{14}	3.0×10^{13}	0.15
Rabbit C64	B	0.19	1.3×10^{14}	1.2×10^{13}	0.09
Rabbit C167	B	0.55	3.7×10^{14}	4.9×10^{13}	0.13
Rabbit C173	B	0.58	3.9×10^{14}	3.4×10^{13}	0.09

* Antibody eluted from the immunoadsorbent with the NaCl-phosphate buffer, pH 3.0.

‡ All protein was assumed to be IgM antibody. The protein content was measured by the Lowry method.

§ A molecular weight of 900,000 was used.

‖ The antibody was diluted sufficiently to provide C'1a fixation and transfer values of 2.7 to 16.7×10^9 molecules in the individual experiments.

In previous studies, the number of C'1a molecules fixed has been proportional to the number of IgM antibody molecules added to a constant amount of erythrocyte antigen, i.e., the dose-response curve had a slope of one (4, 21). This relationship was tested for rabbit IgM anti-benzenearsonate using dilutions of pool 1 antibody (Table I). The graph of C'1a molecules fixed as a function of the number of antibody molecules demonstrated a straight line with a slope of 1.18 (Fig. 3). The C'1a-fixing properties of rabbit IgM anti-R are like those of all other IgM antibodies tested in this way (4, 21).

Binding of ^{131}I-Labeled Antibodies to R-SRBC.—Since only a fraction of the

IgM antibody molecules fixed C'1a, it was essential to consider the possibility that the nonfixing antibody molecules did not bind to the sheep erythrocytes under the conditions of the test. The first stage of the C'1aFT test was repeated using ^{131}I-labeled anti-R so that the proportion of antibody molecules bound to the R-SRBC could be measured. While the antibody to cell ratio affected the percentage of labeled antibody bound to the R-SRBC, as much as 88% of the

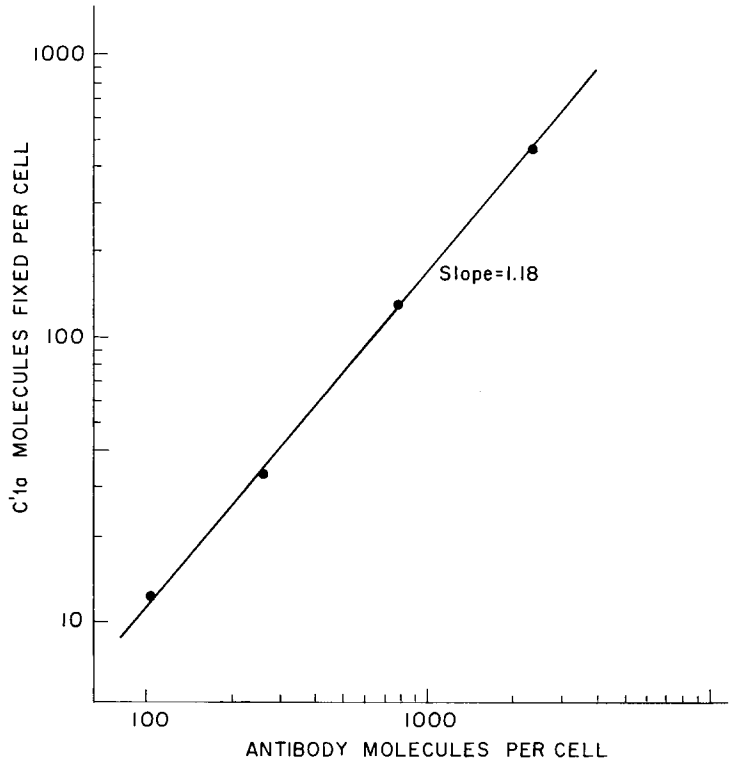


FIG. 3. Fixation of C'1a as a function of antibody concentration. Dilutions of anti-R were added to 2×10^8 R-SRBC. The number of antibody molecules is calculated from the mg of protein added.

radioactivity remained with the cells after four washings with low ionic strength VB–saline-sucrose. Normal (uncoupled) SRBC incubated with the same amount of labeled antibody bound only 2% of the protein added.

C'1a fixation was compared directly with percentage of radioactivity bound in an additional experiment. Samples of ^{131}I-labeled IgM antibody were incubated with 8×10^9 R-SRBC and with the same number of control (uncoupled) SRBC in 2 ml total volume for 10 min at 37°C.

0.1 ml was then removed from each mixture, brought to 1.0 ml with VB–saline-sucrose, and assayed by the standard C'1aFT test. Duplicate 0.4 ml samples of the original cell-antibody mixture were incubated with 0.1 ml C'1a for 10 min at 30°C. The cells were then washed four times with 4.5 ml of VB–saline-sucrose and the amount of radioactivity retained on the erythrocytes was determined. The number of IgM molecules bound to the R-SRBC was five times the number of C'1a molecules fixed (Table II). While the C'1a fixed was only 15.5% of the IgM antibody added, under these conditions 74% of the IgM molecules were bound to the R-SRBC. When the efficiency of rabbit IgM

TABLE II

Comparison of Antibody Binding and C'1a Fixation Using ^{131}I-Labeled Rabbit IgM Anti-Benzenearsonate Antibody

	R-SRBC	Normal SRBC
Antibody binding:		
Net cpm of ^{131}I-labeled antibody added	81	76.0
Net cpm of erythrocytes after washing	60	1.5
Proportion of antibody bound to erythrocytes	0.74	0.019
C'1a fixation:		
Antibody molecules added*	3.5×10^{11}	3.3×10^{11}
Antibody molecules bound‡	2.6×10^{11}	0.07×10^{11}
C'1a molecules fixed	0.56×10^{11}	0.009×10^{11}

For the R-SRBC:

$$\frac{\text{C'1a molecules fixed}}{\text{Antibody molecules added}} = \frac{0.56}{3.5} = 0.155 \qquad \frac{\text{C'1a molecules fixed}}{\text{Antibody molecules bound}} = \frac{0.56}{2.6} = 0.21$$

* Calculated assuming all the protein present was IgM antibody (molecular weight 900,000).

‡ (Antibody molecules added) × (proportion of antibody bound to erythrocytes).

anti-R was calculated for those molecules bound to the R-SRBC, 21% of the IgM antibody molecules fixed C'1a.

Effect of Purification on C'1a-Fixing Capacity.—Partial destruction of C'1a-fixing capacity during the IgM antibody purification would cause an apparent difference when the number of C'1a-fixing molecules was compared with the number of antibody molecules.

The magnitude of this effect was determined by comparing C'1a-fixing activity and antibody protein at each step of the purification (Table III). Antibody was isolated from anti-R globulin depleted of IgG by DEAE-cellulose chromatography.

The amount of antibody left on the immunoadsorbent after elution was estimated by a microcolumn method using 1.2–1.5 mg of ^{131}I-labeled (IgG-

TABLE III

Preservation of C'1a Fixing Activity During Antibody Purification

	Serum V404			Serum V407		
	Antibody purification		Microcolumn assay using ^{131}I-labeled protein	Antibody purification		Microcolumn assay using ^{131}I-labeled protein
	Mg protein	C'1a fixing molecules	Net cpm	Mg protein	C'1a fixing molecules	Net cpm
Starting globulin*	301	36.5×10^{14}	597,221	90	11.5×10^{14}	398,834
Protein not adsorbed by the immunoadsorbent	284	1.9×10^{14}	592,692	80	1.5×10^{14}	388,926
Antibody eluted with 1 M NaCl, 0.05 M phosphate, pH 3.0	8.9	9.5×10^{14}	1,675	2.1	2.2×10^{14}	3,047
Antibody eluted with 0.1 N HCl	4.4	4.0×10^{14}	1,092	2.0	1.6×10^{14}	1,494
Protein retained on immunoadsorbent	—	—	1,762	—	—	2,408
Recovery of C'1a fixing molecules in antibody fractions		$\frac{13.5}{36.5} = 37\%$			$\frac{3.8}{11.5} = 33\%$	
Recovery of adsorbed radioactivity			$\frac{2,767}{4,529} = 60\%$			$\frac{4,541}{6,949} = 65\%$
Calculated recovery of antibody‡ (100% − loss during adsorption-loss during elution)	$100\left(1.00 - \frac{1.9}{36.5} - \frac{1,762}{4,529}\right) = 56\%$			$100\left(1.00 - \frac{1.5}{11.5} - \frac{2,408}{6,949}\right) = 52\%$		

* IgG removed by DEAE chromatography.

‡ The amount lost during adsorption measured by C'1a-fixing molecules in protein not adsorbed. The amount lost during elution measured by radioactivity retained on immunoadsorbent.

depleted) globulin.[2, 3] This allowed direct determination of the protein-bound radioactivity retained on the adsorbent. It was assumed that the eluted antibody had the same specific activity as that retained on the immunoadsorbent.

The recovery of C'1a-fixing capacity reflected the recovery of antibody

[2] Hoyer, L. W., W. E. Vannier, and L. Renfer. Antibody elution from hapten-cellulose immunoadsorbents: the effects of hapten structure, pH and salt concentration. *Immunochemistry*. In press.

[3] The radioactivity per mg of IgG-depleted globulin was much greater than that of the purified antibody when measured directly (V404 globulin, 28,150 cpm/mg; V404 antibody, 3300 cpm/mg; V407 globulin, 18,900 cpm/mg; V407 antibody, 4800 cpm/mg) or calculated from the data of Table III. Similar differences in specific activity have been found when whole serum was iodinated before isolation of protein fractions (22). The ratio of cpm (microcolumn assay) to mg of antibody isolated (large scale antibody purification) varied without pattern for a different reason. These two experiments were done on different scales and included

protein during the steps of the purification. Antibody is most effectively purified with this benzenearsonic acid–cellulose immunoadsorbent at protein-adsorbent ratios such that most, though not all, of the antibody is adsorbed in the first passage through the adsorbent column.[2] 5 and 13% of the antibody (measured by C'1a-fixing antibody) was not adsorbed from the anti-R globulins. Incomplete elution caused an additional loss of 40 and 35% of the adsorbed antibody. Although losses occurred at both steps of the purification, we recovered 56 and 52% of the IgM antibody in the starting globulins. The recovery of C'1a-fixing

TABLE IV

C'1a Fixation by IgM Anti-Benzenearsonate Eluted with Different pH Buffers

Source of purified IgM antibody*	pH of isolation‡	Antibody concentration§	Antibody molecules/ml‖	C'1a molecules fixed/ml	C'1a molecules fixed / Antibody molecules
		mg/ml			
Rabbit V569	6	0.28	1.8×10^{14}	1.7×10^{13}	0.09
	3	0.35	2.3×10^{14}	2.2×10^{13}	0.09
Rabbit V570	6	0.28	1.8×10^{14}	1.7×10^{13}	0.09
	3	0.42	2.8×10^{14}	1.7×10^{13}	0.06
Rabbit V404	3	0.11	7.3×10^{13}	1.1×10^{13}	0.15
	1–2	0.042	2.8×10^{13}	3.8×10^{12}	0.14
Rabbit V407	3	0.081	5.4×10^{13}	8.7×10^{12}	0.16
	1–2	0.053	3.5×10^{13}	4.1×10^{12}	0.12

* All rabbits immunized by schedule B.

‡ Antibody eluted at pH 6 and pH 3 with 0.05 M phosphate, 1 M NaCl. Antibody eluted at pH 1–2 with 0.1 N HCl.

§ All protein was assumed to be IgM antibody. The protein determined by Kjeldahl nitrogen analysis with 6.25 conversion factor (rabbits V569 and V570) or by Lowry protein determination (rabbits V404 and V407).

‖ A molecular weight of 900,000 was used.

activity (37 and 33%) was, therefore, two-thirds of that calculated for the IgM antibody.

In other experiments, the fraction of antibody molecules that would fix C'1a was found to be independent of the pH of antibody isolation (Table IV).

Electrophoretic Characterization of the IgM Antibody.—Functional heterogeneity among the IgM antibody molecules could produce a deficiency in the

different manipulations (gel filtration, concentration, and two dialyses during antibody isolation) which could affect the amount of antibody isolated. The specific activity of antibody isolated from ^{131}I-labeled anti-R globulin was not affected by the pH at which the antibody was eluted.

number of C'1a molecules fixed. Evidence of heterogeneity was obtained by preparative electrophoresis of 11.2 mg of IgM antibody (pool 2, Table I). The protein concentrations of the fractions obtained were determined (optical density at 280 mμ) and the C'1a-fixing capacities measured. A comparison of

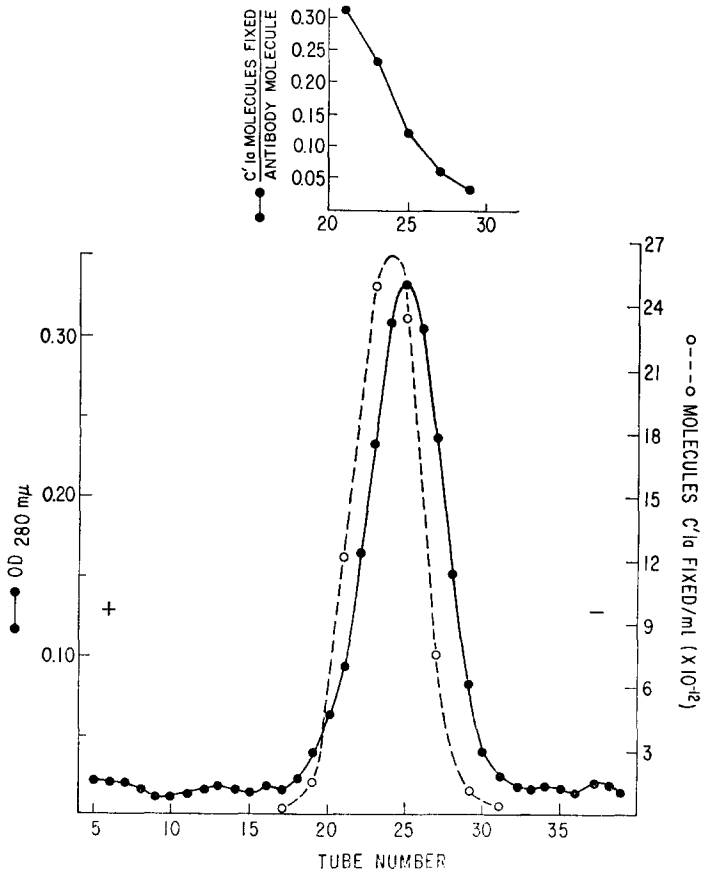


FIG. 4. Preparative electrophoresis of purified rabbit IgM anti-benzenearsonate antibody. The solid line represents optical density at 280 mμ, and the broken line represents the number of molecules of C'1a fixed/ml. Above this data is plotted the calculated ratio of C'1a molecules fixed to antibody concentration in each tube. The anode is to the left.

the two values (Fig. 4) demonstrates relatively greater C'1a fixation by the more negatively charged IgM molecules. The ratio of C'1a molecules to antibody molecules is also given in Fig. 4. The number of antibody molecules in each fraction was calculated from the optical density at 280 mμ using $E^{1\%}_{280\,m\mu} = 13.2$ for rabbit IgM and assuming that all the protein present was antibody

with a molecular weight of 900,000. The ratio fell from 0.31 in the most anodal fraction to 0.03 in the most cathodal fraction having sufficient protein to permit an accurate estimation of antibody content.

DISCUSSION

Quantitative immunologic methods may emphasize different manifestations of an immune interaction. Most determinations require the identification of the antigen-antibody complex, either in solution or as a precipitate. Another measure of the number of antigen-antibody complexes generated by a serum is the number of C'1a molecules fixed and then transferred using the C'1aFT test. As the number of C'1a molecules fixed is directly proportional to the amount of IgM antibody added (4, 21), the test is an effective quantitative measure of antibody. Moreover, in the two systems previously studied (rabbit IgM anti-Forssman antibody and human IgM anti–A isoagglutinin), the number of C'1a molecules fixed was the same as the number of IgM antibody molecules determined by quantitative precipitation or by antigen binding (5, 6).

The properties of specifically purified rabbit IgM anti–R antibody were different, however, when measured using the C'1aFT test. Although the anti–R was immunologically active, some molecules which combined with antigen failed to fix C'1a. Evidence for the activity of the antibody included the following characteristics: over 95% of the molecules were present in antibody-antigen complexes formed in antigen excess and examined by free boundary electrophoresis, 88% were bound by hapten-coupled erythrocytes, and 75% precipitated in quantitative precipitin assay. Nevertheless, C'1a fixation was associated with only 21% of the number of antigen-antibody complexes formed as measured by radiolabeled antibody bound to the cell.

While some loss of C'1a-fixing capacity was detected during the purification procedure, this was not sufficient to account for the difference between complexes formed and C'1a molecules fixed. This important control demonstrated that the low ratio of C'1a molecules fixed to antibody molecules was not an artifact of the method of antibody purification.

Most of the experiments were done with antibody prepared from pools of rabbit serum, but similar results were obtained when purified preparations from eight individual rabbits were assayed. The C'1a-fixing capacity of the purified antibody was also the same when two different schedules of immunization were compared.

Previous studies with the C'1aFT test have used erythrocyte surface antigens for comparison of C'1a-fixing capacity and antibody content on a molecular basis (5, 6). There may be significant differences in the antibody-antigen-complement relationships when these intrinsic antigens are compared with small haptenic groups coupled to proteins of the erythrocyte membrane. The size of the binding sites, the strength of the antigen antibody bond, and the

number of antigenic sites may be significantly different. Moreover, the importance of antigenic heterogeneity may differ when the two kinds of antigens are compared.

Our findings with hapten coupled directly to erythrocyte membrane protein were confirmed using tanned SRBC coated with R-BSA. Parallel experiments demonstrated 80% of the C'1a fixation with RBSA-tanned SRBC when compared with R-SRBC sensitized with the same quantity of purified IgM antibody.

Although the IgM antibody preparations appeared to contain only trace contamination when examined by ultracentrifugation, immunoelectrophoresis, and disc electrophoresis in acrylamide gel, partial separation of fractions with different C'1a-fixing activity could be obtained by preparative electrophoresis. The concentration of C'1a-fixing antibody molecules in the anodal region suggests that there are at least two different kinds of IgM molecules.

Since complement-fixing capacity is associated with heavy chain characteristics (2), the separation of C'1a-fixing molecules from non-C'1a-fixing molecules may be due to a previously unrecognized μ chain heterogeneity. In this case, the difference in electrophoretic mobility may be related to the difference in functional properties; both caused by slight changes in the composition or structure of the antibody μ-chain.

It has been suggested that there are two types of human IgM anti-A antibodies, and that some individuals have only nonhemolytic "naturally occurring" IgM anti-A (23). While this has not been investigated in detail, the data we have obtained would support such an hypothesis. The electrophoretic and functional heterogeneity of guinea pig IgG antibodies (24) demonstrates a well-studied example of the kind of reactivity we have noted for rabbit IgM. Little is known of the relationships between the different antibody types within each class. It remains to be determined if the production of different antibody types within a class is regulated by the form in which the antigen is presented, the physical properties of the antigen, or the stage of the immune response. The heterogeneity of rabbit IgM anti-benzenearsonate offers a well-defined system for further investigation of these questions.

SUMMARY

The C'1a-fixing properties of purified rabbit IgM anti-benzenearsonate antibody were determined. When tested with sheep erythrocytes to which hapten had been coupled by diazo linkage, the number of C'1a molecules fixed was 21% of the number of IgM antibody molecules bound to the erythrocyte surface. This was not due to loss of C'1a-fixing capacity during the purification procedure. Preparative electrophoresis of the antibody concentrated C'1a-fixing molecules in the anodal region so that antibody fractions with greater C'1a-fixing capacity were obtained. The demonstration that C'1a fixation is a

property of a subpopulation of IgM molecules provides evidence for previously unrecognized μ-chain heterogeneity.

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Note Added in Proof.—P. H. Plotz, H. Colten, and N. Talal (March 1968. *J. Immunol.*) have reported the existence, in mice, of a noncomplement-fixing subpopulation of IgM molecules.

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