ISOLATION OF RABBIT IgA ANTIHAPTEN ANTIBODY AND DEMONSTRATION OF SKIN-SENSITIZING ACTIVITY IN HOMOLOGOUS SKIN*

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Several classes of well characterized immunoglobulins are known in human and other animal species. Although antibodies of all classes of immunoglobulins combine with the antigen, they differ greatly in their activity in various immunologic phenomena which have requirements in addition to the combination of antigen and antibody.

Only antibodies of a few immunoglobulin classes have been shown to be active in sensitizing skin. Furthermore, it has been reported that the antibody which sensitizes homologous skin is not necessarily the one which sensitizes heterologous skin. Thus, although human IgA antibody has been associated with reagin or antibody capable of sensitizing human skin (1–5), it did not sensitize guinea pig skin (6, 7). In contrast, human IgG antibody did not sensitize human skin but sensitized guinea pig skin. IgM antibody did not sensitize either skin (6, 7). Similar observations were made with guinea pig antibodies (8, 9) and with mouse antibodies (10–12).

We have previously shown (13) that in rabbits antibody activity against simple haptenic groups is found in at least 3 classes of immunoglobulins, 7S γ-globulin (IgG), 18S γ_1- or β_2-macroglobulin (IgM), and a β_1-globulin (IgA). IgG and IgM antibodies were isolated and characterized for physical, chemical, and immunologic properties (14, 15). Rabbit IgA antibody, which was found in only a few antisera, was shown to be similar to human IgA globulin in physical and antigenic properties relative to the other immunoglobulins. The results of gel filtration and ultracentrifugation indicated that rabbit IgA antibody is associated with a 9S globulin and possibly also with a 7S globulin (13).

In the present study, the presence of 9S and 7S IgA antibody in rabbits was shown clearly by isolating them by gel filtration and chromatography from specifically purified antibody against the *p*-azobenzenearsonate group (R_p). In

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addition to physical and antigenic properties, skin-sensitizing activity in homologous (rabbits) and heterologous species (guinea pigs) was studied with 9S IgA, 7S IgA, IgM, and IgG antibodies. The results indicated that, in homologous species, the skin-sensitizing activity was shown by 7S IgA antibody, but not by 9S IgA antibody. The activity of IgG antibody was very low and IgM antibody did not sensitize rabbit skin. In contrast, IgG antibody was the most active in sensitizing skin of the heterologous species.

Materials and Methods

Antisera.—Antisera against the *p*-azobenzenearsonate (R_p) group were obtained from rabbits immunized by intravenous injections of a *p*-azobenzenearsonate-bovine γ-globulin conjugate (16). Injections and bleedings were repeated weekly. Sera were collected during a period of 6 to 12 months, pooled, and used for the preparation of antibodies. Horse antiserum against rabbit globulin (horse anti-RG) (13), sheep antiserum against the whole macroglobulin fraction of normal rabbit serum (sheep anti-RMG) (14), and guinea pig antiserum against normal rabbit IgG (GP anti-IgG) (14) were the same antisera as those used in previous studies. Goat antisera against Fab fragment, Fc fragment, H chain, or L chain of normal rabbit IgG were prepared by repeated injections of each antigen (5 to 10 mg each time) incorporated in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit). Each antiserum was made specific to the antigen prior to use by mixing with the counterpart fragment or chain.

Purification of Antibody.—Anti-R_p antibodies were purified by the use of an immunoadsorbent prepared by coupling R_p groups to an insoluble polymer of rabbit serum albumin (R_p-poly RSA) by the method described previously (17). In brief, antibodies adsorbed on the R_p-poly RSA were eluted with 0.3 M benzenearsonate at pH 8 and the hapten was removed by dialysis. The antibody solution was concentrated by pervaporation in the cold. From 3 liters of pooled serum 530 mg of purified antibody was obtained by the use of 1 g of R_p-poly RSA.

Gel Filtration.—Gel filtration on Sephadex G-200 was carried out according to the method of Gelotte et al. (18) using a Tris buffer of pH 8.0 containing 0.5 M NaCl (14).

Diethylaminoethyl (DEAE)-Cellulose Chromatography.—The same buffer systems as described previously for the separation of IgM and IgG antibody (14) were used for the separation of IgA antibody and IgG antibody.

Fractionation of Normal Rabbit Globulin.—The crude globulin fraction from normal rabbit serum, obtained by precipitation at 50% saturation of ammonium sulfate, was fractionated by elution from DEAE-cellulose with a Tris-phosphate buffer system. The fraction eluted with a buffer at pH 8.4 and a conductance of 1.7×10^{-3} reciprocal ohms contained only IgG and is referred to as *normal IgG*. The fraction eluted with a buffer at pH 6.0 and a conductance of 21.8×10^{-3} reciprocal ohms after prior elution at pH 8.4 and a conductance of 2.2×10^{-3} reciprocal ohms, was fractionated further on Sephadex G-200. Two peaks of protein were obtained. The second protein peak from Sephadex contained a number of 7S components which migrated in the γ-, and β-globulin regions upon electrophoresis and is referred to as *normal γ_1-β-globulin*. Proteins in the first peak from Sephadex were refractionated on Sephadex G-200 and the protein in the first peak thus obtained is referred to as *normal macroglobulin*.

Tests for Skin-Sensitizing Activity.—(*a*) A Prausnitz-Küstner (P-K) type test was carried out either in normal rabbits or guinea pigs. Antibody (0.1 ml) (or normal globulin fractions) in 0.15 M NaCl was injected intradermally. After an interval of 24 hr (or 4 hr), a solution of 2% Evans blue in 0.15 M NaCl (2 ml into rabbits and 0.5 ml into guinea pigs) was injected. The dye was injected into the ear vein in rabbits and into the heart in guinea pigs. An antigen (10 μg) in 0.1 ml of 0.15 M NaCl was then injected intradermally at the site of antibody injection. The reaction (blueing at the site) started within a few minutes and developed to

maximum within 30 min. The skin was removed 30 min after antigen injection, placed on a transparent plastic sheet, and the size of the blue spot was measured. (*b*) Passive cutaneous anaphylaxis (PCA) was done essentially according to the method of Ovary (19). Antibody (0.1 ml) in 0.15 M NaCl was injected into a rabbit intradermally. After an interval (incubation period) of 4, 5, or 24 hr, a mixture of 20 mg of antigen and 40 mg of Evans blue in 3 ml of 0.15 M NaCl was injected into the ear vein. The blueing started within a few minutes and developed to a maximum within 30 min. The skin was removed 30 min after the antigen injection and the reaction was judged in the same way as for P-K type test. (*c*) Rabbit serum albumin coupled with R_p groups (R_p-RSA) was used as antigen for the skin reaction. It was prepared by reacting 30 moles of diazotized *p*-arsanilic acid per mole of RSA at pH 9. The The average number of R_p groups coupled to tyrosyl and histidyl residues of RSA was 7.8 moles per mole of RSA, as determined by spectrophotometry (20).

Radioimmunoelectrophoresis (RIE).—Use of this method in the antihapten antibody system was described previously (13, 14).

Determination of Antibody Content by the Use of Immunoadsorbent.—The total antibody content of sera or protein fractions was determined by the adsorption of antibody with R_p-poly RSA. Details of this method were described in a previous paper (14).

Determination of the Amount of IgG in Various Fractions.—This was done by an immunodiffusion technique similar to that of Fahey (21) using guinea pig antiserum against rabbit IgG (GP anti-IgG). This antiserum reacted only with rabbit IgG, but not with IgM or IgA, indicating the absence of any cross-reacting antibodies. An agar layer containing diluted GP anti-IgG was made on a microscope slide. Wells of the same size were made in this layer. Equal volumes of a standard IgG preparation at different concentrations and a test preparation at two different concentrations were applied in wells aligned on the same slide. After diffusion overnight, the slide was washed, dried, and stained with amido black. The IgG concentration in a test sample was determined by the use of reference curves relating the size of the precipitate circle against the concentration of standard IgG. The two values determined at different concentrations of a test sample usually agreed within ±10%.

Reduction and Alkylation of Antibody Preparations.—Reduction was carried out in 0.1 M 2-mercaptoethanol for 40 min at room temperature and then at 4°C for 6 hr at pH 8. The protein concentration during reduction was 2.3 mg/ml. The reduced protein was then alkylated with iodoacetamide at pH 8 and 0°C for 30 min. Excess reagents were removed by dialysis at 4°C. The details were described previously (14).

Ultracentrifugal Analysis.—The analysis was carried out with a Spinco Model E ultracentrifuge, using sodium chloride solution (0.15 or 0.5 M) buffered at pH 8.0 with borate as solvent. The sedimentation constant, $S_{20,w}$, was calculated by conventional methods.

RESULTS

Fractionation of Antibodies.—A purified antibody preparation was obtained from a pool of antisera against *p*-azobenzenearsonate. It contained antibodies of the three immunoglobulin classes, IgG, IgM, and IgA, as determined by radioimmunoelectrophoresis (RIE). As shown in Fig. 1, when this preparation was fractionated on Sephadex G-200, proteins were separated into two peaks, which were pooled as indicated. The first peak contained 18S and 9S components and a small amount of 7S component. The second peak contained 7S component with a small amount of component sedimenting slightly faster than 7S. The first peak portion was refractionated on a Sephadex G-200 column. Most of the proteins this time appeared in a single peak at the position of the

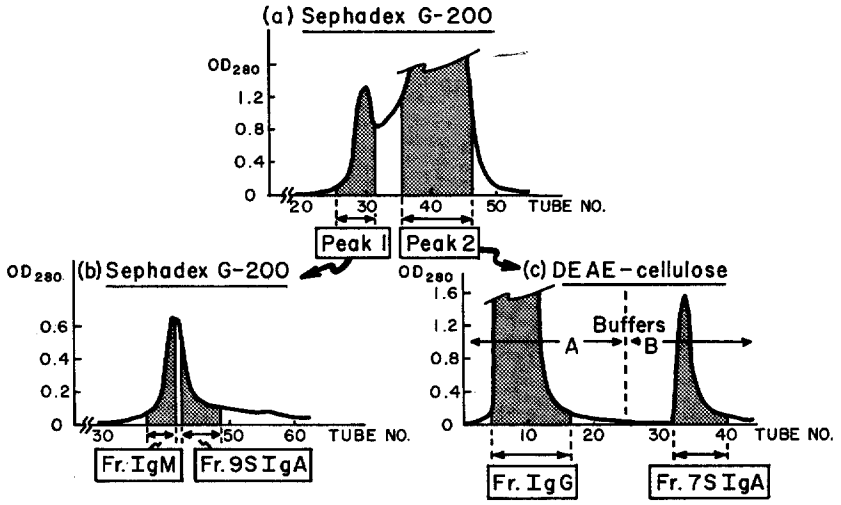

FIG. 1. Fractionation of purified anti-R_p antibodies.

(*a*) Sephadex G-200 (first gel filtration). Purified antibody preparation (270 mg in 4.0 ml) was fractionated on a 3.7 × 78 cm column equilibrated with 0.5 M NaCl buffered at pH 8 with Tris-phosphate (Tris concentration, 0.02 M). Effluent was collected in 9.4 ml portions. Protein recovered in peak 1 was 27 mg (10%) and that in peak 2 was 203 mg (75%). Total recovery of protein was 100%.

(*b*) Sephadex G-200 (second gel filtration). The protein in peak 1 was concentrated and 17 mg (in 2.5 ml) was refractionated on the same column as used for the first fractionation in the same medium. Effluent was collected in 6.3 ml portions. Protein recovered in the earlier portion of the peak (Fr. IgM) was 5.2 mg (31%) and that in later portion (Fr. 9S IgA) was 4.8 mg (25%). Total recovery of protein was 107%.

(*c*) DEAE-cellulose chromatography. The protein in peak 2 (198 mg) of the first gel filtration, was fractionated on 2g of DEAE-cellulose. The initial buffer, A, was Tris-phosphate, pH 8.4 with a conductance of 2.2×10^{-3} reciprocal ohms and the second buffer, B, was Tris-phosphate, pH 6.0, with a conductance of 21.8×10^{-3} reciprocal ohms. Protein recovered in Fr. IgG was 190 mg (96%) and that in Fr. 7S IgA was 8.9 mg (4.5%). Total recovery of protein was 101%.

original first peak. The peak portion was cut into three fractions and the first and third fractions are referred to as *Fr. IgM* and *Fr. 9S IgA*. The second peak of the first gel filtration showed arcs of IgG and IgA on RIE. It was further fractionated on DEAE-cellulose to obtain IgG antibody and a fraction containing IgA antibody. These two fractions are referred to as *Fr. IgG* and *Fr. 7S IgA*, respectively.

Characterization of Each Antibody Fraction.—Table I summarizes the composition of each fraction. All fractions showed a high antibody content, indicating that the amounts of inactive immunoglobulins in each were very small. The

ultracentrifugal patterns and the radioautographs obtained by RIE are shown in Figs. 2 and 3, respectively.

Fr. IgM consisted of a major 18S component (65%) and a minor 9S component (35%) (Fig. 2). Two antibody-active components, IgM and IgA, and a trace of IgG antibody were observed by RIE (Fig. 3). Electrophoresis in agarose (22) indicated that most proteins migrated to the γ_1-region correspond-

TABLE I

Analyses of Components in Each Antibody Fraction

Antibody fraction	Fr. IgM	Fr. 9S IgA	Fr. IgG	Fr. 7S IgA
Antibody components*	IgM, IgA (IgG)	IgA, IgM (IgG)	IgG	IgA, IgG
Size distribution, area % 18S	65	30	0	0
9S	35	64	0	(15)§
7S	0	6	100‡	85
IgG, % ‖	(2.5)¶	9	100	24
Total antibody, %**	92	84‡‡	94	83‡‡

* Determined by RIE. Components in parentheses are trace or minor components.

‡ A small amount of aggregates which sedimented slightly faster than 7S was observed.

§ The component(s) sedimented slightly faster than 7S (7–9S) without forming separate peaks.

‖ Determined by diffusion technique in antibody agar plate with Fr. IgG as standard.

¶ Amount was too small to be determined accurately.

** Determined by adsorption with R_p-poly RSA.

‡‡ Antibody contents of reduced-alkylated preparations were 78% for Fr. 9S IgA, and 72% for Fr. 7S IgA.

ing to the position of IgM. The remaining proteins migrated to the β_1-region corresponding to the position of IgA. The amount of IgG as determined by immunodiffusion in antibody-agar plates was less than 3%.

Fr. 9S IgA consisted of a major 9S component (64%), a minor 18S component (30%), and a small amount of 7S component (6%) (Fig. 2). Three antibody components, IgA, IgM, and IgG, were seen by RIE (Fig. 3). Larger amounts of proteins migrated to the β_1-region and lesser amounts to the γ_1-region upon electrophoresis in agarose. The amount of IgG determined by immunodiffusion (9%) accounted for all the 7S component observed in ultracentrifugation (6%).

When the radioautographs obtained with the above two fractions (Fig. 3) were compared, the IgM antibody arc was stronger and the IgA antibody arc

was weaker for Fr. IgM than the corresponding arcs for Fr. 9S IgA. Thus, the results indicated that the 18S component represented IgM antibody and the 9S component represented IgA antibody in these fractions.

Fr. IgG consisted of a single 7S component essentially and contained only IgG antibody by RIE (Fig. 3).

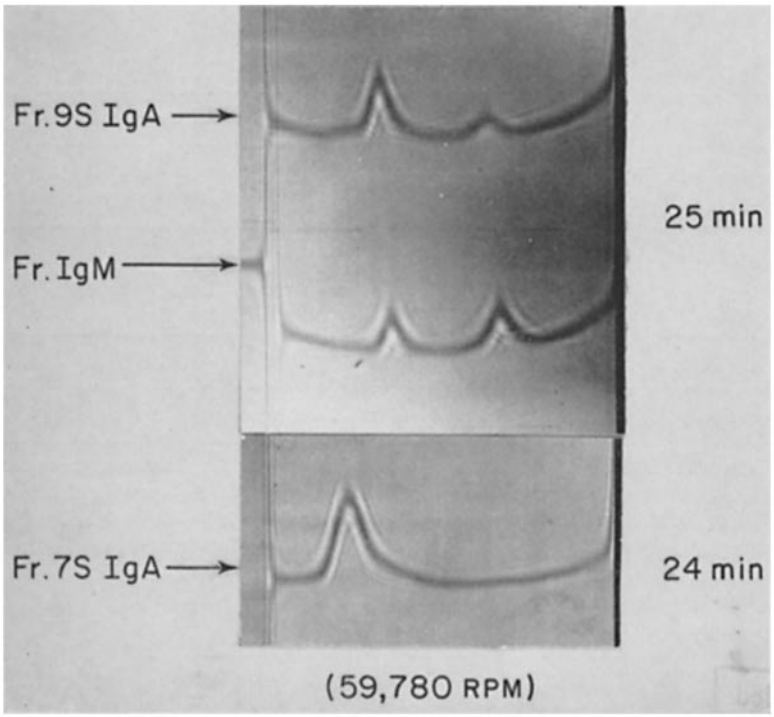

FIG. 2. Ultracentrifugation of the antibody fractions. Fr. 9S IgA was examined at 4.3 mg/ml, Fr. IgM at 4.7 mg/ml, and Fr. 7S IgA at 3.4 mg/ml. Photographs were taken 25 min after the speed reached 59,780 RPM for Fr. 9S IgA and Fr. IgM, and 24 min after for Fr. 7S IgA. The solvent was 0.5 M NaCl (pH 8.0) for Fr. 9S IgA and Fr. IgM, and 0.15 M NaCl (pH 8.0) for Fr. 7S IgA.

Fr. 7S IgA contained a 7S component with a small amount of components sedimenting slightly faster than 7S (Fig. 2). IgA and IgG antibodies were observed by RIE (Fig. 3). In addition to the antibody-active components, a protein migrating to the region of albumin was seen as a faint arc on the stained slide, but this component did not bind the labeled antigen at all. Upon electrophoresis, most of the protein migrated to the β_1-region corresponding to the position of IgA. The remaining protein migrated to the γ_1-region corresponding

to the position of IgG. (The IgG in this fraction showed γ_1-mobility since it was the fraction which was adsorbed more strongly than the rest of IgG on DEAE-cellulose.) It is clear from the data that the sedimentation constant of most of the IgA in this fraction was 7S.

The content of 7S IgA antibody in this fraction was estimated from the values shown in Table I. Since total antibody content was 83% and the content of IgG was 24%, the difference, 59%, represented a minimum value for IgA antibody. If all the components which sedimented slightly faster than 7S component (15%) were assumed to be 9S IgA antibody, the amount of 7S IgA anti-

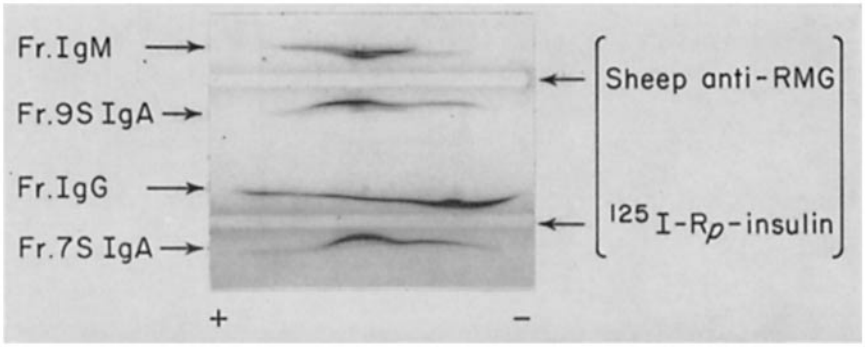

FIG. 3. Radioimmunoelectrophoresis (radioautographs) of the antibody fractions. After electrophoresis of each fraction in agar on a slide, a mixture of sheep anti-RMG and I^{125}-R_p-insulin (2 μg/ml sheep antiserum) was diffused overnight. The slides were washed, dried, and stained. Radioautographs were made by placing a X-ray film in contact with the slides. Protein concentrations of the antibody fractions applied in the wells were 4.7 mg/ml for Fr. IgM, 4.3 mg/ml for Fr. 9S IgA, 3.4 mg/ml for Fr. 7S IgA, and 5.0 mg/ml for Fr. IgG.

body would then be 44% of the total protein. This represented a minimum value since all IgG and 9S components present were assumed to be 100% active. Probably, about 50% of the total protein in Fr. 7S IgA was 7S IgA antibody.

Thus, the presence of the 9S and 7S IgA antibody in rabbits was clearly shown by the isolation of the two fractions, Fr. 9S IgA and Fr. 7S IgA, which contained the 9S or 7S IgA antibody as a major component, respectively. Further support for the presence of the 9S IgA antibody is the fact that Fr. IgM, which was essentially free of 7S component, contained 35% of 9S component and significant amounts of IgA antibody. The 9S component in Fr. IgM can only be attributed to the presence of 9S IgA antibody.

Although a particular pool of anti-R_p serum which was relatively rich in IgA antibodies was selected for this study, the amounts of IgA antibodies in the original antiserum pool appeared to be quite small. It was estimated that only

about 25 mg of 9S and 15 mg of 7S IgA antibodies were present in 530 mg of the purified antibody preparation which had been obtained from 3 liters of the original antiserum. This represented a concentration of 13 μg of IgA antibody per ml of the antiserum.

Antigenic Relationship of IgG with IgA and IgM.—The reaction of IgG, IgA, and IgM with antisera against rabbit globulin, against Fab or Fc fragments of rabbit IgG, and against H or L chains of IgG was studied by immunoelectrophoresis. All three immunoglobulins reacted with horse antiserum against rabbit globulins. IgA and IgM still reacted with the horse antiserum absorbed with the IgG fraction of normal rabbit serum and formed independent arcs, indicating that each immunoglobulin has its own specific determinants. All three immunoglobulins reacted with goat antiserum against the Fab fragment of IgG, but only IgG reacted with goat antiserum against the Fc fragment of IgG. Thus, the specific antigenic determinants of IgG exist on the Fc fragment and the determinants common to IgA and IgM on the Fab fragment. Both IgM and IgG reacted with goat antiserum against the H chain or L chain of IgG indicating the presence of common determinants of IgG on both L chain and Fd part of H chain.

Skin-Sensitizing Activity.—The activity of each specifically purified antibody fraction and the corresponding fraction from normal serum to sensitize homologous (rabbits) and heterologous (guinea pigs) skin was determined.

Skin-sensitizing activity in homologous species: The results of P-K type test are summarized in Tables II and III. As shown in Table II, the only antibody fraction which demonstrated sensitizing activity was Fr. 7S IgA. Typical examples of the skin reaction are shown in Fig. 4. The reaction was much stronger when the antigen was injected at the sensitized sites 24 hr after the intradermal injection of antibody than 4 hr after.

After 24 hr, Fr. 7S IgA showed a strong reaction at a level of 7.5 and 15 μg, whereas neither Fr. IgM nor Fr. 9S IgA showed a positive reaction at a level of 60 μg. Fr. IgG (60 μg) showed a slight or negative reaction. Since Fr. IgM contained some 9S IgA and Fr. 9S IgA some IgM antibody, the observed negative reaction might be due to the possibility that one antibody might inhibit (or block) the activity of the other antibody. This possibility was examined by testing inhibitory activity of Fr. IgM and Fr. 9S IgA against the known active Fr. 7S IgA. As shown in Table II, neither of the two fractions, Fr. IgM or Fr. 9S IgA, inhibited the activity of Fr. 7S IgA. Thus, it appears that these two fractions did not contain any active component capable of being blocked by the other component present. However, normal rabbit serum showed some inhibitory effect on the skin reaction of Fr. 7S IgA. When diluted with normal rabbit serum, 60 μg of Fr. 7S IgA showed a reaction equivalent to that of 15 μg of Fr. 7S IgA diluted with saline.

When the results in Tables II and III are combined, the minimum amount

TABLE II

*Skin-Sensitizing Activity of Rabbit Anti-R_p Antibody in Homologous Skin by P-K Type Test**

Material injected intradermally		4 hr			24 hr				
		Rabbit No.			Rabbit No.				
		41-67	41-68	41-69	41-62	41-61	41-70	41-71	41-73
	μg	*diameter, mm‡*			*diameter, mm‡*				
Fr. 7S IgA	15	6	8	10	13	17	14	15	12
	7.5				12	15			
Fr. IgM	60	*4*	6 (±)	*3*	*4*	*5*	*3*	*3*	*3*
Fr. 9S IgA	60				*4*	*5*	*3*	*3*	*2*
Fr. IgG	60	*3*	7	5 (±)	*4*	*2*	6	5 (±)	6
Fr. 7S IgA	15								
+ Fr. IgM	60	7	5 (±)	8			14	14	11
Fr. 7S IgA	15								
+ Fr. 9S IgA	60						13	14	11
Fr. 7S IgA	15								
+ Fr. IgG	60	6	6	9			13	14	11
Red.-alkyl. Fr. 7S IgA	60	*4*	5 (±)	*3*			*2*	*2*	*2*
Red.-alkyl. Fr. 9S IgA	60	*3*	*3*	*3*			*1*	*3*	*2*
Normal γ_1-β-globulin§	60	*2*	*4*	6			*2*	*3*	*1*
Normal Macroglobulin§	60	*2*	7	*4*			*3*	*3*	*2*
Normal IgG§	60	*4*	*5*	*1*			*2*	*3*	*1*

* Test samples in 0.15 M NaCl (0.1 ml) were injected intradermally. After 4 or 24 hr, rabbits were injected intravenously with 2% Evans blue (2 ml) and then intradermally with 10 μg of R_p-RSA (0.1 ml) at each site of the previous injection. The skin reaction was judged 30 min after the antigen injection. The reaction was negative when RSA was used instead of R_p-RSA as control.

‡ Average diameters of blue spots. Italics indicate the spots which were judged as negative.

§ These were obtained by chromatography on DEAE-cellulose and by gel filtration.

of Fr. 7S IgA required for sensitizing activity was about 4 to 8 μg. Since the 7S IgA antibody content in this fraction was about half of the total protein, the minimum amount of 7S IgA antibody required for P-K type reaction in rabbits appears to be about 2 to 4 μg. That the activity was due to IgA antibody and not due to IgG antibody with γ_1-mobility (present in significant amounts in this fraction), was shown by the use of antisera against papain fragments of IgG. When Fr. 7S IgA was mixed with the goat anti-rabbit Fab serum prior to intradermal injection, the skin reaction was markedly inhibited in comparison

TABLE III

*Effect of Different Anti-Rabbit Serum on Skin-Sensitizing Activity of Fr. 7S IgA in P-K Reaction in Homologous Skin**

Material injected intradermally	Rabbit No.		
	41–96	41–97	41–98
μg	*diameter, mm‡*		
Fr. 7S IgA 15	14	13	8
" 7.5	12	12	*3*
" 3.8	10	8	*3*
" 1.9	*3*	5 (±)	*3*
Fr. 7S IgA 30 + goat anti-Fab	12	6	*4*
" 30 + goat anti-Fc	18	13	*4*
" 30 + normal goat serum	16	12	5 (±)
Goat anti-Fab	*3*	*3*	*2*
Goat anti-Fc	*3*	*3*	*1*
Normal goat serum	*2*	*1*	*2*

* Intervals between the injections of antibody and of antigen was 24 hr.

‡ See footnote ‡ in Table II.

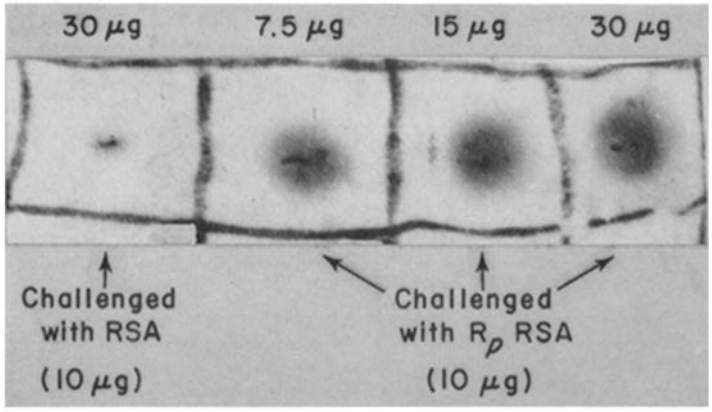

FIG. 4. Skin-sensitizing activity of Anti-R_p Ab Fr. 7S IgA was shown by the P-K type test in homologous (rabbit) skin. Antibody fractions were injected intradermally in μg units in 0.1 ml as indicated in the figure. After 24 hr, Evans blue (40 mg in 2 ml) was injected intravenously, then, the antigen, R_p-RSA (10 μg in 0.1 ml), was injected intradermally at the sites of antibody injection. In the control, 10 μg of RSA instead of R_p-RSA was injected.

to a control mixed with a normal goat serum. However, the goat anti-rabbit Fc antiserum did not inhibit the reaction at all although the antiserum contained enough antibody to precipitate all the IgG antibody present. Since anti-Fab serum reacted with both the IgA and the IgG, whereas anti-Fc serum reacted

TABLE IV

*Skin-Sensitizing Activity of Rabbit Anti-R_p Antibody in Homologous Skin by PCA Test**

Material injected intradermally		4 hr			5 hr				24 hr		
		Rabbit No.			Rabbit No.				Rabbit No.		
		41-93	41-94	41-95	41-50	41-51	41-52	41-53	41-90	41-91	41-92
	μg	*diameter, mm‡*			*diameter, mm‡*				*diameter, mm‡*		
Fr. 7S IgA	30	9	9	7					19	20	16
	20				10	12	10	8			
	15	11	0	8					16	16	8
	10				8	10	4	tr			
	7.5	tr	0	tr					15	16	0
	5				0	9	tr	0			
	3.8	0	0	0					12	10	0
	2.5				0	0	tr	0			
Fr. IgM	100				0	0	0	0			
	30								0	0	0
Fr. 9S IgA	30								6	0	0
Fr. IgG	120	0	0	0					12	11	0
Fr. 7S IgA	30										
+ Fr. IgG	120	7	0	tr					18	22	16
Fr. 7S IgA	30										
+ Normal IgG	120	9	9	8					18	21	16
Normal IgG	120	7	0	0					0	0	0
	100				0	0	0	0			
Normal macroglobulin	100				0	0	0	0			

* Test samples in 0.15 M Nacl (0.1 ml) were injected intradermally. After 4, 5, or 24 hr, rabbits were injected intravenously with a mixture of 20 mg of R_p-RSA and 40 mg of Evans blue in 3 ml 0.15 M NaCl. The skin reaction was judged 30 min after the antigen injection. The reaction was negative when RSA was used instead of R_p-RSA as control.

‡ See footnote ‡ in Table II.

only with the IgG, it is clear that IgA antibody was responsible for the skin-sensitizing activity. Since the other two fractions, which contained 9S IgA antibody, did not show any activity, it is concluded that 7S IgA was the active component in this fraction.

In controls, IgG fraction and whole macroglobulin fractions of a normal rabbit serum, and a 7S fraction containing normal IgA did not give any positive

reaction. When the rabbit skin was injected with 30 μg of antibody fractions and challenged after 24 hr with RSA instead of R_p-RSA, all showed negative reactions (Fig. 4).

Thus, in the P-K type test, only the 7S IgA antibody sensitizes rabbit skin. No activity is shown by the IgM, 9S IgA, or IgG antibody.

TABLE V

*Skin-Sensitizing Activity of Rabbit Anti-R_p Antibody in Guinea Pig Skin by P-K Type Test**

Material injected intradermally		Guinea pig No.		
		1	2	3
	μg	*diameter, mm‡*		
Fr. IgG	30	20	14	12
	10	17	12	9
	3.3	12	11	8
	1.1	13	11	5 (±)
Fr. 7S IgA	30	12	13	*4*
	10	13	11	*3*
	3.3	9	8	*4*
Fr. IgM	30	6	9	*4*
	10	*4*	*3*	*4*
	3.3	*4*	*3*	*4*
Normal IgG§	30	*3*	*3*	*3*
Normal γ_1-β-globulin§	30	6 (±)	*3*	*3*
Normal macroglobulin§	30	*5*	*4*	*4*

* Test samples in 0.15 M NaCl (0.1 ml) were injected intradermally. After 25 hr, guinea pigs received injections of 2% Evans blue (0.5 ml) into heart and then of 10 μg R_p-RSA (0.1 ml) intradermally at each site of previous injection. The skin reaction was judged 30 min, after the antigen injection. The reaction was negative when RSA was used instead of R_p-RSA as control.

‡ See footnote ‡ in Table II.

§ See footnote § in Table II.

Results of the PCA test are shown in Table IV. The active fraction was also Fr. 7S IgA and no activity was shown by Fr. IgM and Fr. 9S IgA. The Fr. IgG showed very low activity. As also observed in the P-K type test, the PCA reaction was much stronger when antigen was injected 24 hr rather than 4 or 5 hr after the intradermal injection of antibody. The end point was 10 to 20 μg for Fr. 7S IgA (therefore 5 to 10 μg 7S IgA antibody) in the 4 hr test and 4 to 8 μg (2 to 4 μg 7S IgA antibody) in the 24 hr test. In the 24 hr test, the activity of 120 μg of IgG antibody was equivalent to that of about 2 μg of 7S IgA antibody (4 μg of Fr. 7S IgA). The small degree of activity does not seem to be due to the presence of the 7S IgA antibody contaminating Fr. IgG because no IgA arc was detected by RIE (<0.5% IgA present). IgG antibody inhibited

the reaction by Fr. 7S IgA at 4 hr but not at 24 hr, at which time the activity of the IgG antibody itself was manifested.

Skin-sensitizing activity in heterologous species (guinea pig): The activity in guinea pigs was tested by the P-K type test with an interval of 24 hr between antibody and antigen injections. As shown in Table V, the activity was shown by IgG antibody (Fr. IgG). The activity shown by Fr. 7S IgA was much less

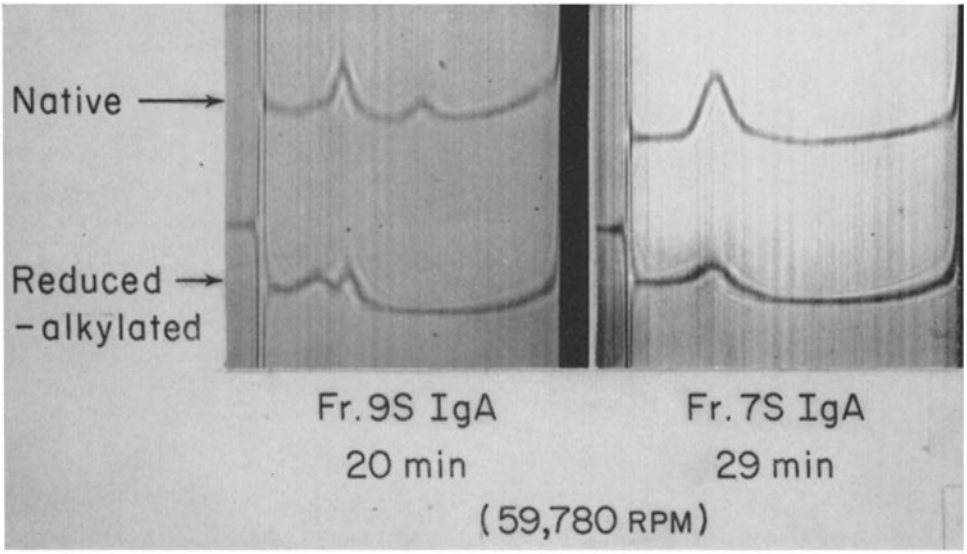

FIG. 5. Ultracentrifugation of native and reduced-alkylated antibody fractions. Protein concentrations examined were: 4.3 and 2.1 mg/ml for native and reduced-alkylated Fr. 9S IgA, and 3.4 and 2.1 mg/ml for native and reduced-alkylated Fr. 7S IgA, respectively. Photographs were taken at 20 min for Fr. 9S IgA, and at 29 min for Fr. 7S IgA after the speed reached 59,780 RPM.

than that of IgG antibody and probably was due to IgG antibody (24%) in this fraction. Fr. IgM was not active.

Effect of Reduction and Alkylation on 9S and 7S IgA Antibody.—Ultracentrifugation patterns for reduced and alkylated Fr. 9S IgA and Fr. 7S IgA are shown in Fig. 5. Both had been treated with 0.1 M 2-mercaptoethanol and then with iodoacetamide. For Fr. 9S IgA, the treatment caused no significant change in 9S peak (Fig. 5), but the 18S peak disappeared completely with a simultaneous increase in the 6S peak. Essentially no change occurred in Fr. 7S IgA. In RIE, the IgA arc in Fr. 9S IgA did not change after the treatment, but the IgA arc in Fr. 7S IgA changed its shape partly. Both arcs bound I^{125}-R_p-insulin strongly. Adsorption experiments with R_p-poly RSA showed that most of the reduced-alkylated products retained antigen-binding activity (Table I, foot-

note‡‡). As shown in Table II, in the P-K type test in homologous skin, both Fr. 9S IgA and Fr. 7S IgA did not show any activity after treatment.

DISCUSSION

Presence of an antibody component in rabbit serum which is similar to human IgA in physicochemical properties, was reported previously (13). In this study, fractions containing rabbit IgA antibody as a major component were isolated by fractionating a specifically purified antibody preparation against *p* azobenzenearsonate. Immunochemical and ultracentrifugal analyses of the fractions clearly showed the presence of IgA antibody in two molecular forms, 7S and 9S. Rabbit IgA antibody was shown to be similar to human IgA in the following aspects: (*a*) IgA antibody migrated faster than most of the IgG in electrophoresis although the mobility of rabbit IgA seemed to be larger than that of human IgA; (*b*) IgA antibody was adsorbed more strongly than most of the IgG on DEAE-cellulose; (*c*) IgA antibody existed in at least two molecular forms, one with a sedimentation constant of 7S and the other 9S; and (*d*) The IgA antibody molecule had antigenic determinants common to IgG as well as determinants specific to IgA.

Rabbit IgA antibody was also similar in its biological function to human IgA antibody. Human skin-sensitizing antibody was shown to be associated with IgA (1–5, 23). The rabbit IgA antibody was also found to have skin-sensitizing activity in homologous skin.

The skin-sensitizing activity in homologous skin was tested by a Prausnitz-Küstner (P-K) type reaction and by the PCA reaction. Only 7S IgA antibody sensitized rabbit skin. In addition to the fact that only the 7S IgA antibody rich fraction (Fr. 7S IgA) showed the activity, the association of sensitizing activity in homologous skin with IgA antibody was supported by the fact that antiserum against the Fab fragment of rabbit IgG, which cross-reacted with the IgA, inhibited the activity of Fr. 7S IgA, whereas no inhibition was shown with antiserum against the Fc fragment, which did not react with the IgA. This is similar to the elimination of the sensitizing activity of reagenic sera by removal of IgA by a specific anti-IgA serum as shown by Fireman et al. (1).

It was noted that the reaction was considerably stronger in both the P-K type test and the PCA test when the interval between the antibody injection and the antigen injection was 24 hr rather than 4 or 5 hr. The minimum amount of antibody required for the positive reaction was about the same in both tests.

Thus, the presence of skin-sensitizing antibody in rabbits was clearly shown and the active component was identified as the 7S IgA antibody. Failure to demonstrate skin-sensitizing activity with rabbit antibody (in homologous skin) in previous investigations by other workers (19) was probably due to the lack of, or insufficient concentration of IgA antibody in the antisera used. Frequency of detecting significant amounts of IgA antibody in rabbit antihapten

sera was very low. In our previous report (13), we found 2 antisera against *p*-azobenzenearsonate which contained IgA antibody out of 6 examined. However, in a later survey of many more antisera, IgA antibody was detected in only 15% of antisera, and of these only a few per cent contained significant amounts of IgA antibody. The limit for detection of the antihapten IgA antibody by radioimmunoelectrophoresis was roughly in the range of a few μg antibody/ml of serum. Even the selected pooled serum used for the present study contained only about 13 μg of IgA antibodies (9S and 7S) per ml of serum. A minimum of 2 to 4 μg of 7S IgA antibody (in 0.1 ml) was required to provoke a PCA or P-K type reaction even when the incubation period before injection of antigen was 24 hr. However, if the PCA reaction was carried out as usual with an incubation period of 4 to 5 hr, a minimum amount of 5 to 10 μg was required. In addition, with an unfractionated antibody preparation, or with the antiserum itself, other antibodies, especially IgG antibody present in much larger amounts, would compete for antigen with the active 7S IgA antibody and block the reaction. Also, normal rabbit serum inhibited the reaction to some extent even in the P-K type test with the 24 hr incubation period, probably by a mechanism in which normal IgA competed with IgA antibody for skin fixation (2, 8, 24–26). Thus, it is rather difficult to demonstrate the skin-sensitizing activity of rabbit antiserum especially by a PCA test with a short incubation period (4 to 5 hr) as usually performed. It is preferable to extend the incubation period to 24 hr to detect the activity. By that time, the inhibition by other antibody components is minimized, although some inhibition by normal serum component still persists.

In contrast to the results in homologous skin, only the IgG antibody sensitized heterologous (guinea pigs) skin when tested by the P-K type test. The IgM and the 9S IgA antibody did not show any activity in both homologous and heterologous skin. The contrasting activity shown by the 7S IgA and the IgG antibody in homologous and heterologous skin and the absence of activity in IgM antibody in both species is in line with the observations with human IgA, IgG, and IgM antibodies (6, 7). Similar observations were made in other species (8–12).

Human IgA exists in monomer form (7S) and various polymer forms (ranging from 8 to 18S) (27). Experiments by gel filtration or ultracentrifugation of skin-sensitizing antibody in combination with immunochemical analyses located most of the sensitizing activity on IgA polymers rather than on IgA of monomer form (1, 4, 23, 28), although the association of some activity with IgA monomer was not excluded. In contrast, in the rabbit antibody system, the activity was found only with 7S IgA antibody but not with 9S IgA antibody.

When the rabbit 7S IgA antibody was treated with a reducing reagent and an alkylating reagent, the skin-sensitizing activity was lost without a significant change in its sedimentation rate and in antigen-binding activity. It was shown

that human IgA polymers transform to the 7S monomer by treatment with 0.1 M mercaptoethanol (29–31). On the other hand, the skin-sensitizing activity of allergic serum was destroyed by treatment with a reducing reagent (32). However, these observations are not enough to indicate that the loss of the activity is solely due to the dissociation of a polymeric IgA antibody to the monomer form. It may be due to some other alteration as we observed with rabbit 7S IgA antibody. Moreover, it was noted that under the same conditions the rabbit 9S IgA did not dissociate to smaller units, while IgM antibody completely dissociated to its 6S subunits.

Note added in proof.—Zvaifler and Becker reported recently in abstract (Zvaifler, N. J., and Becker, E. L., Rabbit passive cunaneous anaphylaxis, *Fed. Proc.*, 1965, **24**, 677) on the PCA-active antibodies in early sera from immunized rabbits. The maximum intensity was obtained at 72 hr of incubation. Although they did not identify the active component(s), its electrophoretic mobility, sedimentation properties, and sensitivity to mercaptoethanol suggest that the active component may also be an IgA antibody.

SUMMARY

Multiple antibody components of rabbit antisera against *p*-azobenzenearsonate (R_p) were studied with respect to their globulin nature and skin-sensitizing activity. IgA antibody was characterized by isolating two IgA-rich fractions from a specifically purified antibody preparation. Examination of these fractions showed that IgA antibodies existed in two molecular forms, one with a sedimentation constant of 7S and the other 9S. Skin-sensitizing activity was examined by a P-K type test and a PCA test with R_p-rabbit serum albumin in homologous (rabbit) species. Only the 7S but not 9S IgA antibody sensitized rabbit skin. IgM antibody showed no activity and IgG antibody showed very low activity. In contrast, only IgG antibody was active in the P-K type test to sensitize a heterologous species (guinea pig). None of the antibodies of other classes showed sensitizing activity in heterologous skin.

The 7S IgA antibody lost its sensitizing activity upon reduction and alkylation, although no change in its molecular size could be observed. The loss of sensitizing activity was not due to the destruction of antigen-binding activity since the treated 7S IgA antibody retained this activity as shown by radioimmunoelectrophoresis and by binding to the specific immunoadsorbent. The 9S IgA antibody was more resistant to these treatments than the IgM antibody and showed no indication of dissociation. The treated 9S IgA also retained antigen-binding activity.

Both the P-K type and PCA reactions were considerably stronger when the interval between injections of antibody and antigen was 24 hr rather than 4 to 5 hr.

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