PHYSICAL PROPERTIES OF ANTIBODY TO BOVINE SERUM ALBUMIN AS DEMONSTRATED BY HEMAGGLUTINATION*

By ALBERT A. BENEDICT, Ph.D., RONALD J. BROWN, and RAJALAKSHMI AYENGAR

(From the Department of Bacteriology, University of Kansas, Lawrence)

(Received for publication, August 31, 1961)

The heterogeneity of antibody as determined by a variety of physical, chemical, and biological methods has been well established. Since the demonstration of high molecular weight horse antipneumococcal antibody (1), numerous studies have shown electrophoretic, ultracentrifugal, and chromatographic differences in human and various animal antibodies (2, 3). Generally rabbit and human anti-erythrocyte antibodies are characterized as electrophoretically slow migrating γ-globulin of low molecular weight (7S) and low anionic binding, and fast migrating γ-globulin of high molecular weight (19S) and high anionic binding (2, 3). Although most studies have been restricted to anticellular antibodies, Wassermann antibodies (4), skin-sensitizing antibodies (5), rheumatoid factor (6), and anti-thyroid auto-antibodies (7), have been shown to belong to the high molecular weight class of antibodies. Certain of these antibodies have been associated with the duration of immunization. For example, 19S horse anti-pneumococcal antibody formed early in immunization is followed by the production of low molecular weight antibody on prolonged antigenic stimulation (8). More recently Stelos (9) has reported early rabbit hemolysin to be mostly a γ-1 with a sedimentation rate of 19S and that a γ-2 hemolysin associated with 6S protein to appear later in immunization. Employing partition chromatography, Porter (10) and Humphrey and Porter (11) found in the early stages of production that anti-protein rabbit antibodies were in the slow moving fractions, and that on further immunization antibody appeared in faster moving fractions. There is, however, a dearth of information of this kind with regard to antibodies to soluble protein antigens. The present study describes the electrophoretic, ultracentrifugal, and chromatographic separation of early and hyperimmune rabbit anti-bovine serum albumin antisera employing the passive hemagglutination (HA) method. Evidence is presented that rabbit anti-protein antibodies are similar to anti-cellular antibodies with respect to their sequential synthesis.

* This investigation was supported by research training grant 2E-158 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

Materials and Methods

Antisera.—Rabbits weighing 3 to 4 kg were immunized with recrystallized bovine serum albumin (BSA), pentex. For production of primary (early) sera, rabbits were given 3 intravenous inoculations of 40, 30, and 20 mg BSA, respectively, at 2-day intervals. Bleedings were made 6, 10, and 17 days following the last injection. Late sera were obtained from these rabbits by intravenous injection of 40 mg of BSA 27 days following the primary series, and were bled 3, 6, and 14 days later. Hyperimmune sera were obtained by giving another group of rabbits 5 intravenous inoculations of 40 mg BSA each, 4 days apart, and a similar series repeated 1 month following the last injection. 9 and 14 days following the second series, intraperitoneal injections of 1 mg BSA in Freund's adjuvant were given. Four months following the intraperitoneal injection 30 mg BSA were given intravenously and bleedings were made when booster titers were elevated.

Lots of BSA used early in this study produced antisera which gave at least 2 precipitin bands in double diffusion in agar (12) and in micro-immunoelectrophoresis (13); however, most of the sera used were produced against lots of BSA which revealed only a single band in gel diffusion and immunoelectrophoresis. Individual rather than pooled sera were studied.

Hemagglutination.—Heat-inactivated sera, adsorbed 1 to 3 times with washed sheep red blood cells, were titrated in 2-fold serial dilutions in 0.5 ml amounts. Some modifications were made of the methods of Boyden (14) and Stavitsky (15) for the preparation of BSA-sensitized tanned sheep red blood cells. The minimum amount of BSA to give optimal sensitization of cells was found to be from 30 to 100 μg for each 1.0 ml of 2.5 per cent tannic acid–treated cells. Following sensitization the cells were washed at least three times with 1.3 per cent normal rabbit serum in pH 7.2 phosphate buffer. Lower hemagglutinin (HAN) titers were obtained with cells inadequately washed. The HA patterns were judged according to Stavitsky (15). Porter (16) had noted that primary and secondary sera produced qualitatively different red cell agglutination patterns and in the present study similar differences were observed. Some primary sera produced ragged edged 4+ reactions rather than complete coating of the bottom of the tubes, usually produced with hyperimmune sera. Also, titers of primary sera invariably dropped following prolonged storage at −20°C. The specificity of the agglutinin in the following fractions was determined by the HA inhibition test (15).

Quantitative Precipitin.—Total precipitable protein was determined either by dissolving the washed precipitates in 0.25 N acetic acid and recording the absorption at 277 mμ in a Beckman DU spectrophotometer (17), or by the Folin reaction (18).

Starch Electrophoresis.—Except for minor modifications, the method of Kunkel and Slater (19) was used. Separation of serum proteins was made using pH 8.6 veronal buffer, 0.1 ionic strength. A constant current of 20 ma was applied for 24 hours at 5°C. Following electrophoresis, the starch bed was cut into 1 cm segments and each segment was eluted with 2 to 4 ml of buffered saline. Hemagglutinin activity of starch eluates usually decreased rapidly following storage at 5°C or at −20°C; thus, when possible, HA was performed within 24 hours following elution. The cause of the HAN lability of these fractions is being studied.

Ultracentrifugation.—Zone ultracentrifugation in a sucrose density gradient using a Spinco model L centrifuge and a swinging bucket rotor was performed according to the method of Brakke (20) modified by Fudenberg and Kunkel (21). Sucrose concentrations ranging from 10 per cent at the top of the plastic tube to 40 per cent at the bottom were made by either layering or use of a mixing device. The procedure given in the above reference (21) was then followed. Serum diluted with an equal volume of saline and cleared at 5,000 RPM for 30 minutes was layered in a volume of 0.5 ml over the sucrose. Centrifugation was carried out for 18 hours at 35,000 RPM. Fractions were collected in 0.8 to 1.0 ml volumes by making a small hole in the tube about 2 to 3 mm from the bottom. A small hardpacked amber-colored gelatinous

pellet was recovered last by rapid washing of the tube with saline and resuspension of the pellet in 0.2 ml of saline.

Analytic studies were kindly performed by Dr. Robert Hersh of the Department of Biochemistry. A model E Spinco analytic ultracentrifuge equipped with a schlieren optical system phase plate and temperature control unit was employed. The cells (30 mm) were used at 50,740 RPM.

Chromatography.—The rapid stepwise elution method of Peterson and Sober (22) was used. Diethylaminoethyl (DEAE)[1] cellulose containing 0.91 mEq N/gm was suspended in 0.5 N NaOH, washed in several changes of distilled water until the suspension turned neutral to litmus, and the finer particles decanted off. The sedimented particles were then washed, resuspended in the starting buffer (0.0175 M PO_4^-, Na^+, pH 6.3), and stored in the cold until use. Columns of 1.0 cm diameter and 11.5 to 15.5 cm height were used for 2.0 to 5.0 ml serum. Heat-inactivated immune sera, adsorbed twice with packed sheep red blood cells, were dialyzed against the starting buffer for 18 to 24 hours before applying to the column. Stepwise elution was performed with the following buffers: (*a*) pH 6.3, 0.0175 M sodium phosphate, (*b*) pH 5.7, 0.04 M sodium phosphate, (*c*) pH 5.5, 0.1 M sodium phosphate, (*d*) pH 4.7, 0.4 M sodium phosphate, and (*e*) pH 4.7, 2 M NaCl in 0.4 M sodium phosphate. The effluent fractions were collected in 5 ml volumes. Some of the fractions, after dialysis against distilled water and against the starting buffer followed by concentration through pervaporation at 10°C, were rechromatographed on new DEAE celluose columns of the same size.

EXPERIMENTAL AND RESULTS

Electrophoresis.—In the early phases of this study various hyperimmune sera were selected for the electrophoretic distribution of HAN and precipitin, and considerable distribution in mobility of HAN was noted. In most instances rabbit HAN was distributed throughout the γ- and β-globulins. Fig. 1 illustrates a serum which showed 2 distinct HAN peaks; one associated with γ-2 globulin and one with γ-1 globulin (β-2). The precipitin was located in the γ-2 globulin region and coincided with the peak HAN activity in this region. A mouse anti-BSA ascitic fluid obtained after several months of immunization also showed a similar broad distribution of HAN through the γ- and β-globulins, and similar to rabbit antibody the precipitin was mainly located in the slow moving γ-globulin. For instance, in the slow moving γ-globulin region of the mouse ascitic fluid (fraction 7), the HA titer was 640 and the precipitate had a O.D. reading of 0.069 at 277 mμ; whereas, fraction 16 in the β-globulin with the same HA titer (640) had no precipitate (O.D. = 0.008) as determined by the quantitative precipitin method. It was evident that the HA method measured antibodies over a wide range of electrophoretic mobilities, and detected antibodies which were either not precipitable or were in concentrations insufficient to be detected by the precipitin method used. Most sera showed two poorly resolved peaks similar to Fig. 1; however, the relative heights varied.

The electrophoretic distribution of HAN appeared to be related to the duration of immunization. Fig. 2 illustrates the distribution of agglutinin of hyperimmune and early sera. As noted previously, both sera had HAN in the γ- and

[1] Selectacel, Schleicher and Schuell.

β-globulins; however, the ratio of peak activity in γ-2 globulin/peak activity in β-globulin was higher in hyperimmune sera. These ratios for three hyperimmune sera were 128, 128, and 100, respectively, and for three early sera the ratios were 16, 16, and 20, respectively. Thus, during the early period of immunization a relatively higher concentration of β-globulin HAN was produced.

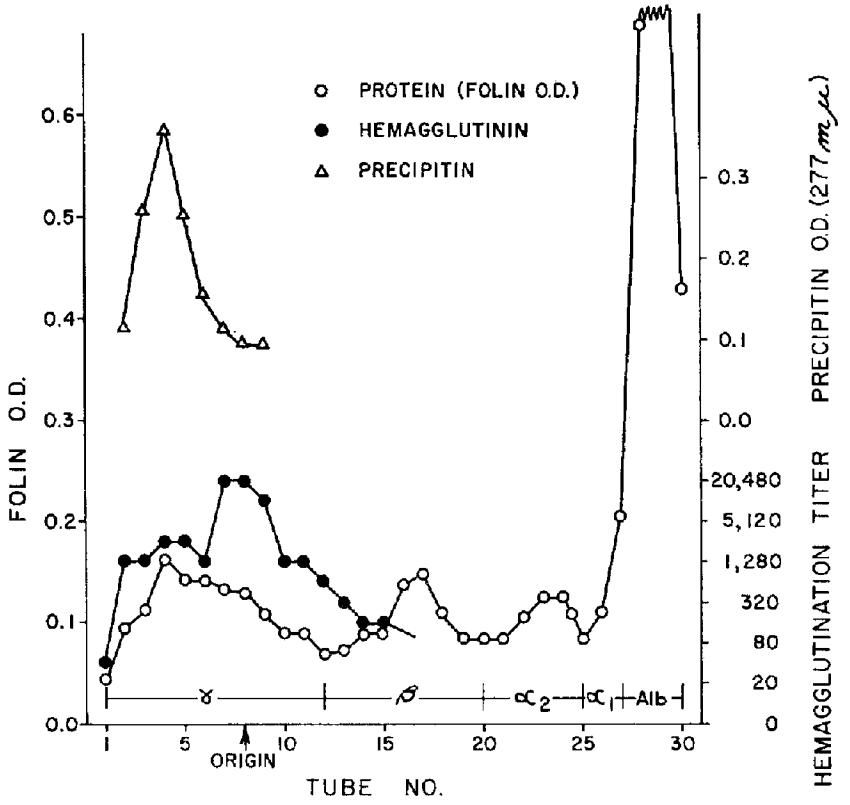

FIG. 1. Starch block electrophoretic distribution of rabbit anti-BSA precipitin and hemagglutinin.

Preliminary studies which chicken anti-BSA antibodies also suggest a relatively high HAN activity in the β-globulin region during early periods of immunization. Ultracentrifugal and chromatographic fractionations corroborated that the anti-BSA HAN differed in physical properties as related to the duration of immunization.

Ultracentrifugal Studies.—The initial studies employed the method of Campbell *et al.* (23). Five ml of late serum diluted with 5 ml of 1 per cent NaCl was cleared at 5,000 RPM for 30 minutes. The serum was centrifuged for 7 hours at 40,000 RPM and partitioned from top to bottom according to Campbell *et al.*

(23). A gelatinous pellet also was recovered. The HAN and precipitin values increased from the top to bottom fractions (Table I). On recycling of fraction 2 (top) and fraction 4 (bottom) at 40,000 RPM for 210 minutes, each fraction again showed increased HAN activity from the top to the bottom of the tube.

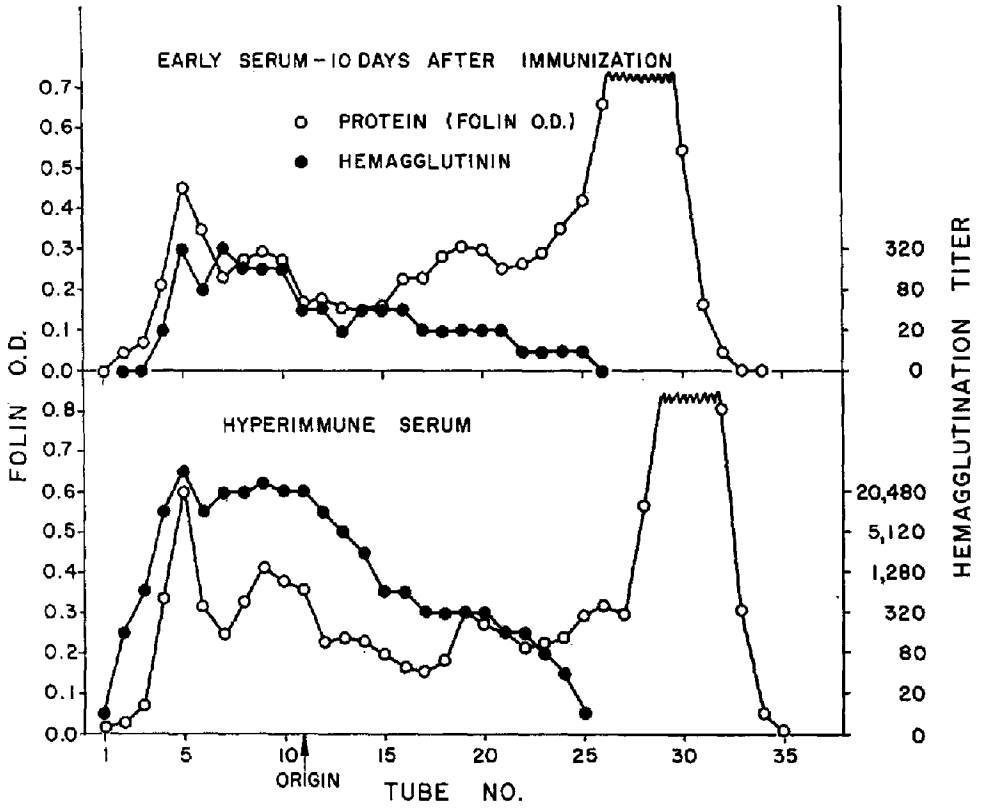

FIG. 2. Starch block electrophoretic distribution of early (top) and hyperimmune (bottom) rabbit anti-BSA hemagglutinating antibody.

Fractions 2 and 4 were also simultaneously starch electrophoresed and both fractions had activity in the γ- and β-globulin regions; however, the top ultracentrifugal agglutinin yielded activity in the slower migrating γ-globulin region (Table II). To obtain better separation of the high and low molecular weight activities sucrose gradients were prepared, and the results are summarized in Tables III and IV. Table III shows fractionation of a hyperimmune rabbit serum, a rabbit serum 5 days following the last primary inoculation, and a chicken anti-BSA serum obtained 8 days following a single intravenous injection of 40 mg of BSA. In this experiment no ultracentrifugal

pellets were recovered. All sera analyzed (early, late, and hyperimmune) had HA activities which had been shown by others (2) to correspond to low and high molecular weight antibodies; however, early in immunization the high molecular weight antibody activity was predominant, and hyperimmune sera had the highest activity corresponding to 7S γ-globulin (Table IV). The ratios of HA titers corresponding to 19S/7S for a rabbit whose serum was obtained 6, 10, and 17 days following immunization (Table IV) were 18.0, 2.1, and 1.8, respectively; thus indicating a drop in 19S activity.

TABLE I

Hemagglutinin and Precipitin in Ultracentrifugal Fractions of Anti-BSA

Fraction*	Hemagglutination titer	Precipitin			
		BSA N added	Protein‡ precipitated	Supernatant test	
				Excess antibody	Excess antigen
		μg	μg		
Unfractionated	6,553,600§				
1 (top)	320	1.7	<25	0	++
		3.5	<25	0	+++
		7.0	<25	0	+++
2	81,920	1.7	116	0	0
		3.5	195	0	0
		7.0	<25	0	++
3	2,621,440	1.7	252	++++	0
		3.5	455	+	0
		7.0	615	0	0
4 (bottom)	5,242,880	1.7	335	++++	0
		3.5	535	++++	0
		7.0	940	++++	0

* Hyperimmune serum centrifuged for 7 hours at 40,000 RPM.

‡ Folin reaction.

§ 1.10 mg antibody N/ml.

Chromatography.—Four distinct protein peaks corresponding to the first four buffers used for elution were obtained (Figs. 3 and 4). The last buffer used, containing 2 M NaCl, failed to elute more protein. In the order of the protein peaks eluted, the fractions have been designated fractions I, II, III, and IV. Early sera had HAN located mainly in fractions I and IV, and the activity in fraction IV was usually the same or greater than in I. One serum chromatographed 10 days following the initial injection of antigen had HAN in fraction III as well (Fig. 3). There was a shift in the relative concentrations of HAN in these fractions following further antigenic stimulation. Hyperimmune sera had agglutinin in all four fractions, with greatly increased activity in I and lowered

TABLE II

Electrophoretic Distribution of Hemagglutinin in Fractions Separated by Ultracentrifugation

Starch electrophoresis fraction No.		Ultracentrifugal fractions*	
		Top	Bottom
1	γ-Globulin	0	0
2		0	0
3		10	0
4		20	0
5 (origin)		80	20
6		80	40
7		80	160
8		80	160
9	β-Globulin	80	320
10		40	160
11		10	160
12		5	40
13		0	20
14		0	10
15		0	10
16			0

* Serum centrifuged for 7 hours at 40,000 RPM.

TABLE III

Distribution of Hemagglutinating anti-BSA Rabbit and Chicken Antibodies in Fractions Separated by Density Gradient Zone Ultracentrifugation

Fraction	Serum		
	Rabbit early	Rabbit hyperimmune	Chicken early
Unfractionated	2,560*	6,553,600	81,920
1 (top)	0	320	40
2	0	2,560	80
3	80	327,680	40
4	40	81,920	320
5 (bottom)	160	2,560	5,120

* Reciprocal of hemagglutinin titer (0 = <1:10).

activity in IV (Fig. 4). In one instance a serum obtained 3 days following booster yielded equal titers in fractions I and IV. This latter serum 17 days following primary injection (before booster) had HA titers of 20 and 160 in fractions I and IV, respectively.

On rechromatography of fractions I and IV, each eluted in the same frac-

tions as before, thus suggesting that HAN with differing characteristics had been separated. Paper electrophoretic analysis of fractions revealed serum proteins similar to those described by Abelson and Rawson (24), and that fraction I contained slow-migrating γ-globulin not found in fraction IV.

Fractions I and IV from an early serum were each subjected to zone ultracentrifugation in a sucrose gradient. Table V shows that low molecular weight HAN resided mainly in fraction I and that the heavier components were associated mainly with fraction IV. For analytic ultracentrifugal studies serum globulins were prepared by repeated precipitation with sodium sulfate (25), concentrated by pervaporation or with the aid of sephadex G-24 (coarse), and

TABLE IV

Distribution of Hemagglutinating anti-BSA Rabbit Antibodies in Fractions Separated by Density Gradient Zone Ultracentrifugation

Fraction	Serum			
	Days following primary injection			Hyperimmune
	6	10	17	
Unfractionated	2,560*	10,240	640	3,276,800
1 (top)	0	0	0	0
2	20	160	80	160
3	10	80	20	640
4	10	80	80	40,960
5	0	0	10	10,240
6	80	40	10	320
7 (pellet)	640	640	320	640

* Reciprocal of hemagglutinin titer (0 = <1:10).

chromatographed. Fraction I of this preparation (HA titer = 6400) consisted of a single homogeneous peak with an s rate of 7.3S. Fraction IV (HA titer = 200) was found to have two peaks; a major component with a sedimentation coefficient of about 4S and a minor component with a sedimentation constant of 20.4S. Since the agglutinin of fraction IV sedimented in a sucrose gradient like a macroglobulin (2), the activity undoubtedly was associated with the 20S component.

Inhibition Tests.—The minimum amounts of BSA required to inhibit agglutination when mixed with chromatographic fractions I and IV were determined. The HA titers of early serum fractions I and IV, hyperimmune serum fractions I and IV, and hyperimmune serum fraction I which had been recycled through DEAE-cellulose were each determined. The last tube showing a 4+ agglutination was considered as 1 HA unit. In the inhibition test, 4 HA

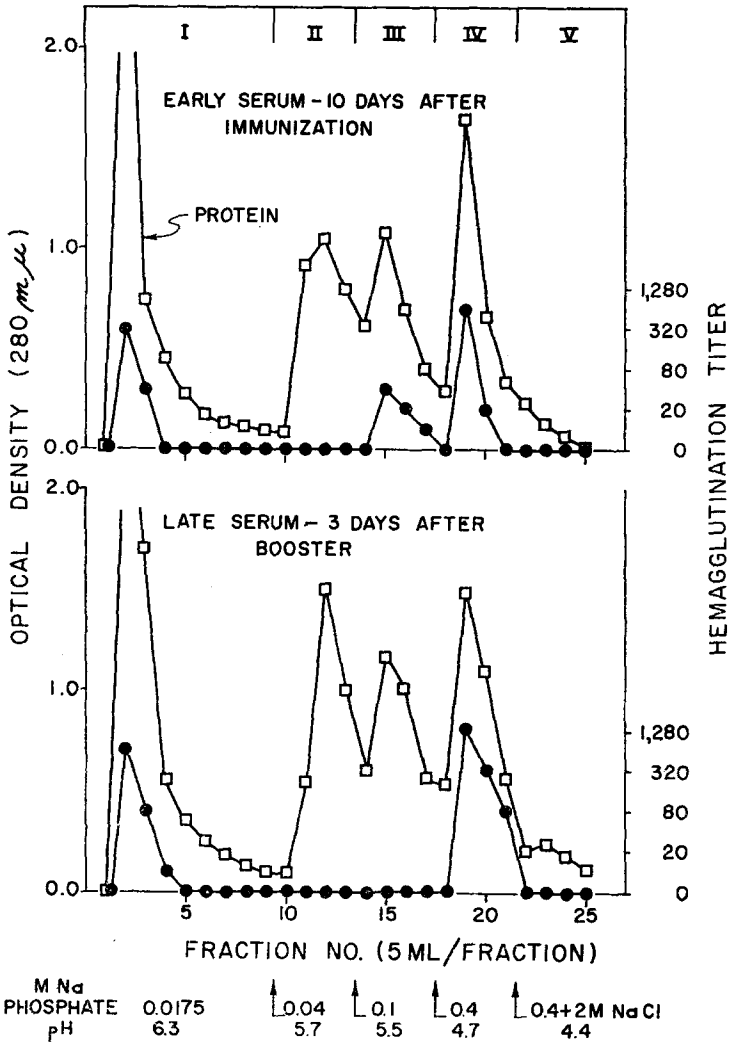

FIG. 3. Stepwise elution from DEAE-cellulose columns of early (top) and late (bottom) rabbit anti-BSA hemagglutinating antibody.

units/0.25 ml were mixed with 0.25 ml of various concentrations of BSA, incubated at room temperature for 10 minutes, and then sensitized red cells were added. Table VI shows that the smallest amount of BSA to partially inhibit HA was the same for early and hyperimmune sera fraction IV and for early serum fraction I. Agglutination by hyperimmune serum fraction I was inhibited with about 200 times less antigen.

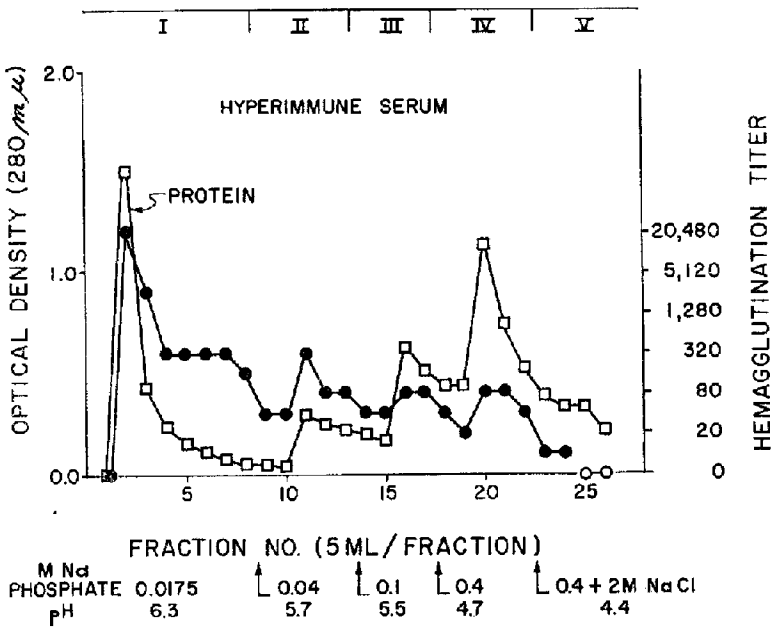

FIG. 4. Stepwise elution from a DEAE-cellulose column of rabbit anti-BSA hemagglutinin

TABLE V

Density Gradient Zone Ultracentrifugation of DEAE-Cellulose-Separated Rabbit Hemagglutinating Antibodies

Fraction	Titer											
	DEAE-cellulose fraction I*						DEAE-cellulose fraction IV					
	5	10	20	40	80	160	5	10	20	40	80	160
1 (top)	±	0	0	0	0	0	0	0	0	0	0	0
2	+++	+	+	0	0	0	+	0	0	0	0	0
3	++	+	0	0	0	0	0	0	0	0	0	0
4	++++	++++	++++	+	0	0	0	0	0	0	0	0
5	+++	+	0	0	0	0	+++	0	0	0	0	0
6	+++	+	+	0	0	0	++++	++++	++++	++	++	0
7 (pellet)	0	0	0	0	0	0	++++	++++	++++	+++	+	0

* DEAE-cellulose fractions I and IV of a serum 10 days following primary injection.

DISCUSSION

It is becoming clear that rabbits may respond to various antigenic stimuli in a similar manner. Stelos (26) reported the production of antibodies to red cell antigens not unlike those reported in the present study, and Kunkel (2) reported that by employing density gradient centrifugation that rabbit anti-

pneumococcal antibodies were present in both the high and low molecular weight fractions.

In response to a protein antigen, at least two antibody fractions have been partially identified; namely, the classical 7S γ-globulin which had the usual low anionic binding capacity (fraction I), and a γ-1 globulin (β-2) with high anionic binding (fraction IV) and associated with a 20S fraction. Early primary sera had a mixture of these two types of antibodies with a relatively high concentration of fraction IV. On further immunization fraction I became the major antibody fraction, although all hyperimmune sera had some fraction IV activity. Also, DEAE-cellulose separation revealed at least 2 more fractions with HAN with intermediate anionic binding, similar to rabbit antibody against capsular polysaccharide of Type III pneumococci (27). Although the density gradient ultracentrifugal studies were highly indicative that high molecular

TABLE VI

Amounts of BSA to Inhibit Agglutination by DEAE-Cellulose-Separated Fractions of Rabbit Antibody

Serum fraction	Minimum amount BSA giving partial hemagglutination inhibition
	μg
Early serum Fraction I	2.0
Early serum fraction IV	2.0
Hyperimmune serum fraction I	0.01
Hyperimmune serum fraction IV	2.0
Hyperimmune serum fraction I, rechromatographed	0.05

weight anti-protein antibodies were synthesized, further evidence must await preparation of more highly purified fraction IV samples. Whether the anti-BSA antibodies associated with the high molecular weight fraction differ from the low molecular weight antibodies in regard to combining capacity, sensitivity to reducing compounds, and other biological properties (2) must be learned. It might be noted here that unlike the cold agglutinins belonging to the macroglobulin class, starch block eluates in the γ- and β-globulin regions, fraction I, fraction IV, and unfractionated sera failed to show increased titers in the cold.

The question remains open whether the cells synthesizing the 19S antibody in early antibody production represent the same types producing the 7S classic antibody found in hyperimmune sera. The intravenous route was used for production of early antibody; possibly the early macroglobulin and 7S antibody were synthesized by splenic cells, and extrasplenic sites were mainly responsible for production of 7S antibody which was predominant following prolonged antigenic stimulation. The kinds of cells involved in the synthesis of the antibodies reported here and elsewhere (2) remain an intriguing problem.

This study was initiated because of the poor correlation between the passive HA titers and the quantitative precipitin antibody N values of numerous rabbit anti-BSA sera. As yet there is no definitive evidence that "non-precipitable" antibody agglutinated BSA-sensitized red cells; however, preliminary evidence suggested that hyperimmune rabbit anti-BSA precipitin migrated mainly as a γ-2 globulin, and that HAN migrated as γ- and β-globulins. Possibly the two methods measured different anti-BSA antibodies, or that because of the high sensitivity and non-specificity of the HA technique (28) a contaminating antigen-antibody hemagglutinating system was detected (29). Although further studies are required to resolve this problem, the presence of a contaminating hemagglutinating system is unlikely. Both high and low molecular weight agglutinins were present 6 days following inoculation as shown by gradient zone ultracentrifugation, and chromatography of 10-day immune sera yielded at least 2 distinct fractions of approximately the same activity. If a minor antigenic contaminant was present in the BSA preparations it is doubtful that the agglutinin associated with the 20S fraction which migrated as a β-globulin, and which was synthesized early in antibody production, was directed towards the contaminant. Pertinent to this problem, adjuvants were not used for production of primary sera.

Future data on the relative combining capacities of the antibody fractions separated may give information relative to this problem. In this regard, fraction I of a hyperimmune serum with a sedimentation rate of 7S apparently had a better combining ability than did a similar fraction from a primary response serum. The formation of antibodies of higher reactivity following continued immunization is well known. Dixon *et al.* (30) reported that the antibody N/antigen N ratios of rabbit anti-BSA and BSA increased from 5 after the first injection to from 8 to 11 after second and third injections. Comparison of the HA inhibition test and precipitate formation cannot be made at this time, since mixtures of antibody and antigen in ratios of extreme antigen excess (according to the precipitin reaction) cause agglutination of sensitized red cells (31). In contrast to fraction I, fractions IV of early and hyperimmune sera were inhibited from agglutinating red cells by the same concentrations of BSA. This might imply that the antibody associated with the 19S fraction, and which decreased in concentration on continued immunization, might not reflect the changing combining characteristics of the 7S antibody. Nevertheless, caution should be employed when comparing antibody concentrations of a immunologically homogeneous system by the passive HA method and other methods of assay.

SUMMARY

Rabbit antibovine serum albumin antisera were fractionated by zone electrophoresis on starch, zone ultracentrifugation in sucrose density gradients, and diethylaminoethyl-cellulose chromatography, and were assayed by the

passive HA technique. Antisera had at least two antibodies: one associated with a fraction characterized as a γ-2 globulin with a sedimentation rate of 7.3S and low anionic binding (fraction I), and one associated with a fraction characterized as a γ-1 globulin (or β-globulin) with a sedimentation rate of 20.4S and high anionic binding (fraction IV). The HA titers of fractions I and IV of early sera were approximately equal. On prolonged antigenic stimulation fraction IV agglutinin decreased in concentration and the relative concentration of fraction I agglutinin increased. Chromatographic analysis of hyperimmune sera yielded 2 additional HA fractions with intermediate anionic binding. These results indicated that rabbits synthesize anti-BSA antibodies similar to rabbit anti-cellular antibodies.

Rabbit anti-BSA precipitin migrated mainly as a γ-2 globulin and the agglutinin migrated with γ- and β-globulins. The evidence suggested that the HA method might measure antibody not measured by the quantitative precipitin technique.

BIBLIOGRAPHY

1. Heidelberger, M., and Pedersen, K. O., The molecular weight of antibodies, *J. Exp. Med.*, 1937, **65**, 393.
2. Kunkel, H. G., Macroglobulins and high molecular weight antibodies, *in* The Plasma Proteins, (F. W. Putnam, editor), New York, Academic Press, Inc., 1960, **1**, 279.
3. Porter, R. R., γ-globulin and antibodies, *in* The Plasma Proteins, (F. W. Putnam editor), New York, Academic Press, Inc., 1960, **1**, 241.
4. Davies, B. D., Moore, D. H., Kabat, E. A., and Harris, A., Electrophoretic, ultracentrifugal, and immunochemical studies on Wassermann antibody, *J. Immunol.*, 1945, **50**, 1.
5. Sehon, A. H., Symposium on Mechanisms of antibody formation, Czechoslovak Academy of Science, Prague, 1961, 79.
6. Franklin, E. C., Holman, H. R., Muller-Eberhard, H. J., and Kunkel, H. G., An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis, *J. Exp. Med.*, 1957, **105**, 425.
7. Pressman, D., James, A. W., Yagi, Y., Hiramoto, R., Woernley, D., and Maxwell, W. T., Rapidly sedimenting properties of specifically precipitating component of a Hashimoto's disease serum, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 773.
8. Kabat, E. A., Immunochemistry of proteins, *J. Immunol.*, 1943, **47**, 513.
9. Stelos, P., Comparative study of rabbit hemolysins to various antigens. I. Hemolysins to beef red cells, *J. Infect. Dis.*, 1958, **102**, 103.
10. Porter, R. R., The fractionation of rabbit γ-globulin by partition chromatography, *Biochem. J.*, 1955, **59**, 405.
11. Humphrey, J. H., and Porter, R. R., An investigation on rabbit antibodies by the use of partition chromatography, *Biochem. J.*, 1956, **62**, 93.
12. Ouchterlony, O., Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion, *Acta Path. et Microbiol. Scand.*, 1953, **32**, 231.
13. Scheidegger, J. J., Une micro-méthode de l'immuno-éléctrophorèse, *Internat. Arch. Allergy*, 1955, **7**, 103.

14. Boyden, S. V., The adsorption of protein on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera, *J. Exp. Med.*, 1951, **93,** 107.
15. Stavitsky, A. B., Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells, *J. Immunol.*, 1954, **72,** 360.
16. Porter, R. J., Studies on antibody formation. Effects of x-irradiation on adaptation for the secondary response of rabbits to bovine γ-globulin, *J. Immunol.*, 1960, **84,** 485.
17. Gitlin, D., Use of ultraviolet absorption spectroscopy in the quantitative precipitin reaction, *J. Immunol.*, 1949, **62,** 437.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193,** 265.
19. Kunkel, H. G., and Slater, R. J., Zone electrophoresis in a starch supporting medium, *Proc. Soc. Exp. Biol. and Med.*, 1952, **80,** 42.
20. Brakke, M. K., Zone electrophoresis of dyes, proteins and viruses in density-gradient columns of sucrose solutions, *Arch. Biochem. and Biophysics,* 1955, **55,** 175.
21. Fundenberg, H. H., and Kunkel, H. G., Physical properties of red cell agglutinins in acquired hemolytic anemia, *J. Exp. Med.*, 1957, **106,** 689.
22. Peterson, E. A., and Sober, H. A., Fractionation of serum protein on anion-exchange cellulose. *J. Am. Chem. Soc.*, 1956, **78,** 751.
23. Campbell, D. H., Sturgeon, P., and Vinograd, J. R., Separation of complete and incomplete Rh antibodies by centrifugation, *Science*, 1955, **122,** 1091.
24. Abelson, N. M., and Rawson, A. J., Studies of blood group antibodies. I. Fraction of anti-A and anti-B isohemagglutinins by anion-cation cellulose exchange chromatography, *J. Immunol.*, 1959, **82,** 435.
25. Kekwick, R. A., The serum proteins in multiple myelomatosis, *Biochem J.*, 1940, **34,** 1248.
26. Stelos, P., Taliaferro, L. G., and D'Alesandro, P. A., Comparative study of rabbit hemolysins to various antigens. III. Chromatographic analysis of Forssman hemolysins induced by various antigens, *J. Infect. Dis.*, 1961, **108,** 113.
27. Askonas, B. A., Heterogeneity of globulins produced by plasma cells, *in* Immunochemical Approaches to Problems in Microbiology, (M. Heidelberger and O. J. Plescia, editors), New Brunswick, Rutgers University Press, 1961, 343.
28. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961.
29. Benedict, A. A., and Briggs, N. T., Separation of psittacosis complement fixing and hemagglutinating antibodies by starch electrophoresis, *Virology*, 1958, **6,** 772.
30. Dixon, F. J., Maurer, P. H., and Deichmiller, M. P., Primary and specific anamnestic antibody responses of rabbits to heterologous serum protein antigens, *J. Immunol.*, 1954, **72,** 179.
31. Ayengar, R., and Benedict, A. A., data to be published.