Antibody Activity in Different Chicken Globulins.* (29823)

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Earlier we reported that chickens given a single injection of bovine serum albumin (BSA) synthesized macroglobulin (19S class) hemagglutinins (HA) in the early phases of antibody production, which was followed by the synthesis of increasing amounts of low (7S class) molecular weight HA(1). Subsequently we reported(2) that primary-response antisera had most of the precipitating antibody associated with 7S (γ-globulin, γ_{ss}) fractions, and that these fractions were inefficient for agglutinating sensitized red cells. Rosenquist and Gilden(3) were unable to detect specific interaction between 19S chicken globulin and BSA by the Farr antigen-binding(4) and precipitin techniques. These methods were used in the present study, and in addition, antibody was identified by radioimmunoelectrophoresis (RIE)(5) and passive hemagglutination. Further evidence was obtained that some specific activity was associated with the macroglobulins, and that antibody presumably of the γ_{1A}-type was also synthesized.

Materials and methods. Primary antibody responses were induced in either adult white leghorn or adult white rock cornish (Kimber Farms Strain K44) hens by a single intravenous injection of either 40 mg of BSA or human serum albumin (HSA) (Pentex) or 140 mg of bovine γ-globulin-*p*-azobenzoate (6). Hyperimmune sera were obtained by giving repeated injections of antigen in complete Freund's adjuvant. The birds were bled 6 days following the last injections. The anti-BSA sera of 30 primary-response and of 18 hyperimmune-response chickens were each pooled, and the globulins concentrated by repeated precipitation with Na_2SO_4(1). These preparations were fractionated by gel filtration on Sephadex G-200 employing a column 4.5 $\times$ 40 cm in size, and 0.1 M Tris buffer in 1 M NaCl (pH 8.0) as the eluting buffer(7). Fractions were assayed for antibody by the tanned-formalized-cell method (8), by double diffusion in high salt agar (8 percent NaCl)(2), by the Farr technique(4), and by RIE(5). Antigens were labeled with I^{131} according to the method of Talmage *et al*(9). The antigen binding capacity (ABC) (γN I^{131} BSA bound/ml serum) was calculated according to Farr(4). For RIE, whole chicken antisera were electrophoresed and rabbit anti-chicken globulin antiserum and labeled antigen were added together to the troughs. Anti-chicken macroglobulin antiserum was made specific by immunization of rabbits with macroglobulin-rich fractions, and by adsorption of the antiserum with 7S globulins. Specific antibody and labeled antigen were detected by autoradiography. Primary anti-BSA sera were fractionated by column chromatography in diethylaminoethyl (DEAE) cellulose as previously reported(1). For this purpose, the following buffers were used for stepwise elution: Fraction A: pH 6.4, 0.1 M sodium phosphate, Fraction B: pH 5.8, 0.2 M sodium phosphate, Fraction C: pH 5.4, 0.3 sodium phosphate, Fraction D: pH 4.7, 0.4 M sodium phosphate, Fraction E: pH 4.4, 2 M NaCl in 0.4 M sodium phosphate. Analytic ultracentrifugation and zone ultracentrifugation in sucrose density gradients were carried out as previously described(1).

Results. Sephadex fractionation. The results of fractionation of primary and hyperimmune anti-BSA globulins are shown in Fig. 1. Analytic ultracentrifugation of the first peak (fraction No. 6) had a major component with an uncorrected sedimentation coefficient of 17.4S and a trace of 7.9S material. The second peak (fraction No. 14) had a single component with an uncorrected sedimentation coefficient of 7.5S. Most of

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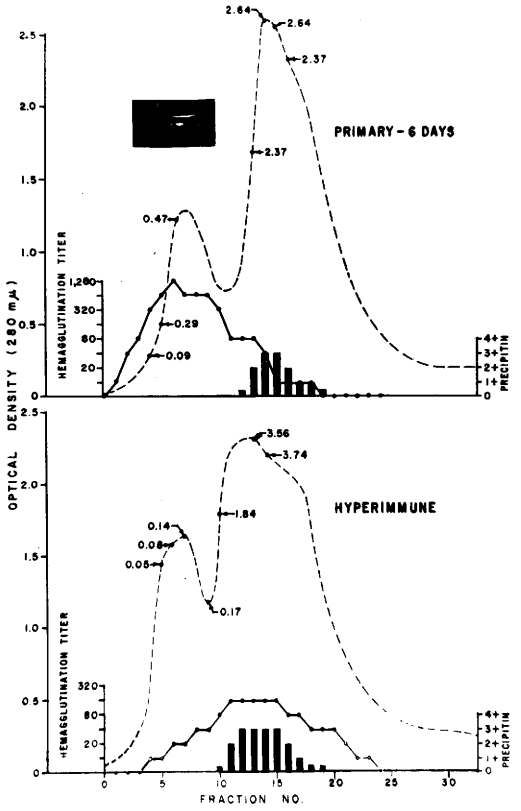

FIG. 1. Fractionation of chicken anti-BSA primary and hyperimmune globulins by Sephadex G-200 gel filtration. Dashed line = protein; solid line = hemagglutinins; bars = precipitins (gel diffusion); arrows with Nos. = antigen binding capacity (γN I^{131} BSA bound/ml fraction); insert = autoradiography of radioimmunoelectrophoresis of (top) primary fraction No. 14, (middle) primary fraction No. 6, and (bottom) normal chicken serum.

the primary HA were eluted in the first peak (Nos. 1 to 10), and hyperimmune HA were in the fractions (Nos. 10 to 20) which consisted of the 7S globulins. This shift in synthesis of high to low molecular weight HA was shown previously by other fractionation procedures(1). Fractionation of primary Sephadex fractions in the first peak (Nos. 3, 6, 8, 10, 12) by zone centrifugation in sucrose gradients revealed rapidly sedimenting HA in each. Trace of 7S HA was found in fraction No. 14. Hyperimmune fractions Nos. 6 and 8 each had small amounts of rapidly sedimenting HA and fraction Nos. 11, 14, and 19, each had only 7S HA. In contrast to the HA, both primary and hyperimmune precipitins and most of the binding antibody (ABC) were located in the lighter fractions. However, primary antisera had low binding activities in the heavier fractions, and these proved to be heavy antibody. The high molecular weight fractions eluted in the same fractions after recycle through fresh gel, and on recycle both HA and binding antibodies were associated with the heavier molecules. When examined by zone ultracentrifugation the binding activity of the primary-response 7S Sephadex fraction (No. 14) sedimented as light antibody, and most of the binding activity of the 19S Sephadex fraction (No. 8) sedimented as heavy antibody (Table I).

DEAE fractionation. Previously it was shown(1) that most of the HA of primary anti-BSA sera appeared in chromatographic fractions C, D and E, and some activity in fraction B. The HA in fractions C, D and E are heavy antibodies, and the HA in hyperimmune sera (fractions A and B) are of the 7S class(1). In the present report, most of the binding antibodies and precipitins of primary and hyperimmune anti-BSA sera were associated with fractions A and B (7S fractions) similar to the findings obtained by Sephadex fractionation (Fig. 1). For example, the ABC values of the peak protein DEAE fractions A, B, C, and D of a primary anti-BSA serum were 5.60, 4.96, 4.34, and 0.11, respectively, and precipitins were detected by gel diffusion in only fractions A and B. The results of zone ultracentrifugation of the peak protein DEAE fractions are shown

TABLE I. Density Gradient Zone Ultracentrifugation of Sephadex-Separated Chicken Anti-BSA Antibodies (Primary Response).

Gradient fraction	% 0.2 μg N I^{131} BSA bound by Sephadex fraction No. 6*	14†
1 (top)	0‡	7.0
2	0	26.3
3	5.1	39.8
4	10.0	94.8
5	24.0	93.3
6	17.1	64.7
7	54.4	18.1
8	97.3	19.1
9	56.4	15.0
10 (bottom)	44.5	7.6

* Heavy (19S) fraction.
† Light (7S) fraction.
‡ Values obtained after subtracting normal serum control.

TABLE II. Density Gradient Zone Ultracentrifugation of DEAE-Cellulose-Separated Chicken Anti-BSA Antibodies (Primary Response).

Gradient fraction	% 0.2 μg N I^{131} BSA bound by DEAE fraction			
	A	B	C	D
1 (top)	3.0*	0	2.4	0
2	.7	0	0	0
3	3.8	.6	2.0	0
4	10.6	24.5	11.9	0
5	56.2	43.1	22.6	0
6	4.1	13.9	11.0	0
7	0	3.3	12.1	0
8	0	8.0	51.4	2.8
9	0	.5	13.5	0
10 (bottom)	0	1.2	9.2	0

* Values obtained after subtracting normal serum controls.

in Table II. Fractions A and B contained the 7S antibodies as detected by the Farr method and fraction C had predominantly heavy antibody although some binding 7S antibody was evident. Low binding activity was found in the heavy fraction of fraction D; however, this is of questionable significance. It was interesting to note that the ABC/mg of protein of fractions A and B were 2.63 and 10.42, respectively. Although 3 to 5 times more globulin is eluted in fraction A than in B, the concentration of antibody is approximately equal in both fractions. This finding is being further investigated.

Radioimmunoelectrophoresis. Fig. 1 shows that the RIE detected the 7S γ-globulin arc in primary fraction No. 14, and that specific binding by antibody resembling γ_1-macroglobulin occurred in the heavier fraction (No. 6). All primary anti-BSA and anti-HSA antisera contained both 7S γ-globulin and γ_{1M}-globulin antibodies (Fig. 2-b, -d). In addition, some primary-response antisera had activity associated with presumably the γ_{1A}-globulin (Fig. 2-d). Hyperimmune antisera had no γ_{1M}-globulin binding, and showed greatly increased 7S γ-globulin activity (Fig. 2-e). A few (5 of 28) hyperimmune antisera were shown to have γ_{1A}-globulin activity. The 7S fractions of primary antisera obtained by zone ultracentrifugation had the expected γ-globulin antibody (Fig. 2-h, -i, -j), and the heavy fraction had γ_{1M}-globulin antibody (Fig. 2-l). There were greatly attenuated 7S arcs and no γ_{1M} antibody in primary anti-BSA and anti-HSA sera which had been treated with 0.1 M 2-mercaptoethanol (ME) (Fig. 2-c). Similar treatment of hyperimmune sera yielded attenuated 7S activity with the appearance of binding activity in the antibody wells (Fig. 2-f). Some ME-treated hyperimmune sera had binding by antibody extending from the well towards the anodal end. Partial inactivation of 7S chicken antibody had been noted previously(1). Employing I^{131}-BSA-*p*-azobenzoate as antigen, RIE of 6-day primary anti-benzoate antisera revealed specific γ_{1A} antibody and possibly a lesser concentration of γ-globulin antibody (Fig. 2-g). No γ_{1M} antihapten antibody has been detected in 12 sera analyzed. The anti-benzoate antibody was found by RIE in zone ultracentrifugal fractions intermediate between the 7S and 19S antibodies. Preliminary experiments demonstrating anti-benzoate by equilibrium dialysis attest to this observation.

Discussion. The tanned-cell method for detection of antibody, particularly in early primary chicken and rabbit antisera, is misleading, since this technique might be more sensitive for detection of heavy antibody than

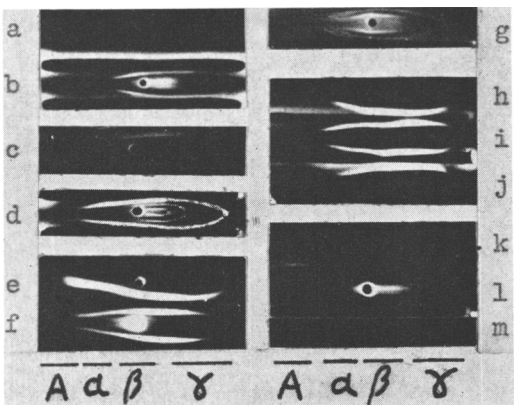

FIG. 2. Radioimmunoelectrophoresis (autoradiography) of chicken antisera. a, normal serum; b, primary anti-BSA; c, primary anti-BSA treated with 0.1 M mercaptoethanol; d, primary anti-HSA; e, hyperimmune anti-BSA; f, hyperimmune anti-BSA treated with 0.1 M mercaptoethanol; g, primary anti-*p*-azobenzoate; h through m, sucrose gradient centrifugal fractions 4 through 9, respectively. Centrifugal fractions 4 through 6 represent 7S γ-globulin and fraction 8 represents γ_{1M} globulin. Homologous I^{131}-labelled antigens and rabbit anti-chicken globulins added to troughs.

the precipitin(2,10), or the Farr methods as reported here. In this connection, 19S rabbit anti-A antibody is 750 times more efficient at agglutinating human group A red cells than 7S antibody(11). Also, we have observed that the binding method demonstrates mainly 7S, and hemagglutination mainly heavier rabbit anti-BSA antibodies. In fact, hemagglutination might measure antibody not measured by the precipitin method, since rabbit anti-BSA precipitins were shown to migrate electrophoretically as γ-2 globulin and the HA as γ-1 and β-globulins(10). Thus, the hemagglutination method is of questionable validity for following the sequential synthesis of 19S and 7S antibodies to certain antigens. Heavy antibody was detected by the Farr method in Sephadex, DEAE, and ultracentrifugal fractions of primary antisera, and the antibody resembled the γ_{1M}-type as detected by RIE of whole primary serums and of fractions containing heavy globulins. The γ_{1M} antibody was not found in hyperimmune sera (Fig. 2-e), and specific rabbit anti-chicken macroglobulin antiserum revealed only γ_{1M} activity.

The γ_{1A}-protein is an immunoglobulin which may sediment in the centrifuge between the 7S and 19S antibodies(12), and in the present study, a few anti-protein antisera had these antibodies. However, the nature of the antigen may play some role in the synthesis of different chicken immunoglobulins. In contrast to anti-BSA and anti-HSA antibodies, all anti-benzoate antisera analyzed showed specific binding by the γ_{1A}-globulin, and in contrast to the predominance of 7S antibody in primary anti-BSA sera, primary chicken anti-bacteriophage ϕX 174 neutralizing antibodies are mainly heavy(13). Furthermore, we have observed that primary chicken anti-bacteriophage precipitins are mainly 19S globulins, and that a high concentration of the 19S antibodies are synthesized following a second injection of bacteriophage.

Rosenquist and Gilden(3) reported no evidence of chicken 19S anti-BSA antibody, and state that "since normal chicken macroglobulin agglutinates latex particles(14) and sheep red blood cells . . . ., it follows that methods used to concentrate macroglobulin may concentrate nonspecific agglutinating factors." All antisera used in the present and previous studies(1,2,10) had been heat-inactivated and adsorbed with normal sheep erythrocytes to eliminate non-specific agglutinins. Furthermore, normal chicken macroglobulin similarly heated or treated with EDTA fails to agglutinate latex particles(14); whereas, specific hemagglutination is not inhibited by EDTA(2). The specificity of the chicken anti-BSA HA has been attested to by inhibition studies and by the characteristics of the antibody response following immunization(1, 2). Finally, in the present report 19S antibody was demonstrated by RIE and by the Farr method, although admittedly the hemagglutination method seems to be more sensitive for detection of 19S antibody in primary antisera.

Summary. Hemagglutination is of questionable validity for following the synthesis of 19S to 7S chicken anti-BSA antibodies, since only small amounts of the major antibody population (7S) are measured in early primary antisera. The 7S antibodies in primary antisera were demonstrated by antigen-binding methods (ammonium sulfate salting out procedure and RIE). Radioimmunoelectrophoretic patterns localized anti-BSA and anti-HSA primary antibody activities in the 7S γ-, γ_{1A}-, and γ_{1M}-globulins; however, only a few of these sera had γ_{1A} activity. No γ_{1M} antibody was detected by RIE in hyperimmune antisera. All anti-azobenzoate antisera had γ- and γ_{1A}-globulin antibodies, but no activity in the γ_{1M}-globulin. The species of chicken antibody synthesized seems to be related to the nature of the immunizing antigen.

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A Micromethod for Determination of Serum Triglycerides.* (29824)

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Any study of the lipid metabolism of individual infants or small animals necessitates the employment of suitable micromethods in order to keep to a minimum the volume of blood serum that is required. Micromethods for determination of cholesterol and phospholipids are available but the original triglyceride method of Van Handel and Zilversmit(1) called for the use of 1 ml of serum although a subsequent report by Van Handel (2) decreased the volume of serum to 0.5 ml. Brody and Carlson(3) independently devised a serum triglyceride method similar in principle to the Van Handel methods(1,2) but which employed 1 ml of serum. In the present study, the method as detailed by Van Handel(2) was scaled down without loss of accuracy so that duplicate determinations for measurement of serum triglyceride concentration can be performed on 0.1 ml serum.

The concentration of reagents and their preparation are essentially those described by Van Handel(2). A 20-80 mesh fraction of Zeolite† was prepared from the 20 mesh grade by partially pulverizing and sifting it. The material was activated by heating at 125°C for 4 hours and placed in a tightly stoppered bottle. It was found that the 20-80 mesh fraction was as reliable as the 80-100 mesh fraction for human serum analyses and easier to handle.

For each serum sample, 0.2 g portions of Zeolite are placed in a series of 15 ml glass-stoppered centrifuge tubes. 1.0 ml of chloroform is added to each tube and the contents agitated on a mechanical mixer.‡ 0.1 ml of serum is placed in each tube, mixed, and an additional 1.0 ml of chloroform is pipetted into each tube. The tubes are stoppered, mixed mechanically for 2 minutes and thereafter occasionally before being allowed to stand overnight. Phospholipids are quantitatively adsorbed on the Zeolite. A standard of purified corn oil(2) or glyceryl tripalmitin in chloroform is treated in a similar fashion.

After standing at least 4 hours, or overnight, the extracts are filtered through Whatman #540 filter paper into stoppered tubes. Duplicate 0.5 ml portions of chloroform extract are placed in 15 × 125 mm test tubes and evaporated to dryness in a water bath. A stream of air is introduced to accelerate the removal of the last portions of solvent.

The glycerides are saponified by heating in the test tubes with 0.5 ml of 0.4% KOH (w/v) in ethanol for 20 minutes at 60-65°. One extra tube with alcoholic KOH is added to the series to serve as a reagent blank. The tubes are stoppered with glass marbles to minimize evaporation and only the bottoms of the test tubes are immersed in the water bath in order to avoid loss of glycerol by volatilization.

After saponification, the tube contents are

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† W. A. Taylor Co., Baltimore, Md.

‡ Turbo-Mixer, TechniLab Instruments, Los Angeles, Calif. or Vortex Mixer, Scientific Industries, Springfield, Mass.