Some Physicochemical and Biological Properties of High Molecular Weight γ and β Globulins in Chicken Sera.* (27195)

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The presence in chicken sera of substances that coprecipitate with antigen antibody complexes has long been recognized(1,2). Recently, Makinodan, Gengozian and Canning (3) have shown these proteins to be γ or β globulins with s rates of approximately 20 S. While these substances appear to resemble certain components of complement, little is known about their biological significance. Serum from normal chickens has also been shown by Rheins, McCoy, Buehler and Burrell(4) to have another unusual property, that of agglutinating latex particles. Experiments were therefore performed to determine whether both of these properties were due to the same high molecular weight proteins and to see whether these substances also have the capacity of reacting with other particulate bodies. The results of these studies show that both functions are associated with the high molecular weight γ and β globulins, and suggest that the agglutination of latex particles by these proteins can be used as a simple tool in studying many of their properties.

Materials and methods. Bloods were obtained at frequent intervals over a period of 18 months from the wing veins of 6 white Leghorn chickens. The blood was permitted to stand at 4°C for 12-18 hours following which the sera were withdrawn. Sera were generally stored at 4°C and used within 2 weeks after preparation. A few studies were carried out on frozen sera that had been kept for longer periods of time. Twice prior to completion of study, the chickens were immunized with human or rabbit γ globulins by a single intravenous injection of 40 mg of the protein. The sera containing antibodies obtained one week later were treated in a similar manner and used for some of the studies.

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Agglutination of latex particles was performed using latex particles 0.81 μ in diameter, kindly given to us by Dr. Jaques Singer as described for the latex fixation test for rheumatoid arthritis(5). Agglutination was read after incubation at 56°C for 2 hours by tapping the tube and noting the character of the agglutinated particles at the bottom of the tube. Tubes were called positive when they contained a coarse sediment of agglutinated particles on the bottom. The first tube showing only a fine cloud on tapping was considered negative. After the initial reading all solutions were resuspended and stored at 4°C for 18 hours, recentrifuged and the titers determined a second time by a similar procedure.

Since the titers with uncoated particles were significantly higher than those with particles coated with gamma globulin, as used in the latex fixation test for rheumatoid factor(5), most of the studies were carried out with latex particles not coated with gamma globulin. To study further the inhibitory effect of gamma globulin and other proteins, experiments were performed with a number of proteins having a wide range of isoelectric points. In these experiments 0.5 ml of a 1% solution of several basic proteins such as gamma globulin, lysozyme, ribonuclease and protamine and more acidic proteins such as albumin and ovalbumin were added to 9.5 ml of the particles prior to addition of the chicken sera. Since the basic proteins frequently caused agglutination of latex particles at pH 8.2, most of the studies with ribonuclease, lysozyme and protamine were performed using pH 7 phosphate buffered saline. This pH was found to yield optimum agglutination by the chicken sera while yielding only small amounts of a fine granular precipitate due to the protein in the control tubes. This could be readily distinguished

from the coarse sediment due to the chicken sera.

Agglutination of sheep cells (SCA) and sensitized sheep cells (SSCA) was performed with a 3% suspension of cells or with cells sensitized with an equal volume of rabbit antisheep cell serum diluted to one-half the basic agglutination titer, according to the method of Ziff *et al.*(6).

Electrophoresis. Zone electrophoresis was performed using starch as the supporting medium and barbital buffer pH 8.6 μ .05(7). Following separation the block was cut in one-half inch segments which were eluted by displacement filtration. Protein concentration was determined by the modified Folin technic(8). The recovered proteins were concentrated by pervaporation prior to further studies.

Analytical ultracentrifugation was performed in a Spinco Model E ultracentrifuge using cells with double sector center pieces.

Preparative ultracentrifugation was performed in a Spinco Model L ultracentrifuge using an SW-39 swinging bucket rotor and a gradient of sodium chloride ranging in concentration from 7 to 21%(9). Protein concentration on the isolated reactions was determined by the modified Folin technic. The location of 7S and 19S peaks was further checked using an antiserum to 7S and 19S human gamma globulins on the fractions obtained from a tube with normal human serum which was fractionated simultaneously.

Results. Agglutination of latex particles. Sera obtained from each of the 6 chickens agglutinated latex particles in dilution ranging from 1:160 to 10,240. Table I shows some representative titers obtained with 3 chicken sera tested at frequent intervals during an 18-month period. During this interval there was some variation in the titers; however none of the sera studied ever failed to agglutinate the particles. The end point of the reaction was easy to read since the sediments were generally coarse and left a clear supernatant solution while negative tubes remained strongly opalescent and had little if any sediment of agglutinated particles. There was little change in titer following 18 hours incubation and only the 2-hour values are listed in Table I and in subsequent studies.

TABLE I. Latex Agglutination Titers of 3 Normal Chicken Sera at Various Intervals over 18 Months.

	Date and titer					
Chick #	6/59	10/59	1/60	3/60	5/60	1/61
1	1,280	10,240	5,120	320	10,240	5,120
2	1,280	10,240	5,120	5,120	5,120	2,560
3	640	5,120	1,280	640	640	1,280

Experiments were then performed to study the effects of adding various proteins to the latex particles prior to addition of the chicken serum. Such proteins are known to coat latex particles(10). Each of the strongly basic proteins listed in Table II such as lysozyme, ribonuclease and protamine caused complete inhibition of agglutination when the reaction was performed at pH 7, while less positively charged substances like gamma globulin, albumin or ovalbumin were less effective.

To determine whether other particulate bodies could be agglutinated by these proteins, similar experiments were performed with sheep cells and sensitized sheep cells. These studies, carried out somewhat less systematically, revealed that these particulate bodies were also agglutinated. However, the titers were lower, ranging from 0 to 112, the titers generally being higher for sensitized cells. The results with these particles were more variable and less reproducible than those obtained with latex particles and only the latter were employed as the method of assay in experiments designed to elucidate the nature of the substances responsible for the agglutination.

Factors influencing agglutination. Agglutination was obtained with fresh sera, as well

TABLE II. Effect of Various Proteins on Agglutination of Latex Particles by 2 Chicken Sera.

		Latex titer	
Protein added	Isoelectric point	Chick 1	Chick 2
None	—	5120	2560
Ovalbumin	4.6	640	640
Albumin	4.6	160	160
RGG	6–8.5	160	160
HGG	6–8.5	80	40
Ribonuclease	9.5	0	0
Lysozyme	11	0	0
Protamine	12	0	0

as sera that had been frozen or stored at 4°C for 2-4 weeks. Frequently, a 2- to 4-fold fall in titer was noted after more prolonged storage and appeared to reflect the amount of spontaneous precipitate formed. The capacity to agglutinate latex particles was always completely abolished by heating at 56°C for 30 minutes and usually by treatment with .01 M EDTA. Agglutinating activity was not affected by inactivation of C'_3 with zymozan, or C'_4 with ammonia(11) or by absorption with washed antigen-antibody precipitates composed of human gamma globulin and rabbit antibody when 1-3 mg of precipitate formed at equivalence was added to 0.5 ml of serum. Similarly, the presence in the sera of antibodies to rabbit gamma globulin and human gamma globulin did not apreciably affect the agglutination of latex particles.

Nature of the proteins responsible. Fig. 1 shows the ultracentrifuge patterns of 2 of the chicken sera which are representative of all of the sera examined at different times. During the early period of centrifugation there appear 2 rapidly sedimenting components, one of which sediments with an s rate of approximately 19S as does one component of normal human serum (Fig. 1), and one of which sediments more rapidly. Fig. 2 shows the electrophoretic pattern obtained with one of the sera and demonstrates that the agglutinating activity was associated solely with the fast gamma (gamma$_1$) and β globulin fractions. Analytical ultracentrifugation of

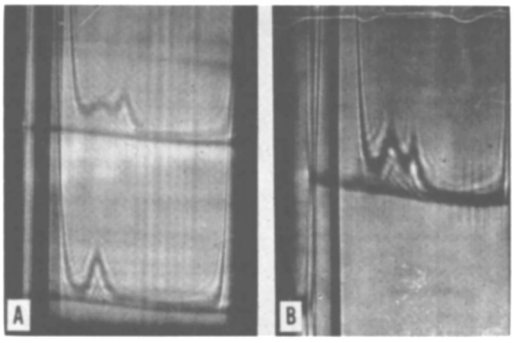

FIG. 1. A. Ultracentrifugal pattern of one normal chicken serum (top) and one normal human serum separated simultaneously—42,040 rpm—48 min. B. Normal chicken serum, 52,640 rpm at 32 min. Each of the chicken sera contains a peak sedimenting ahead of the 19S component.

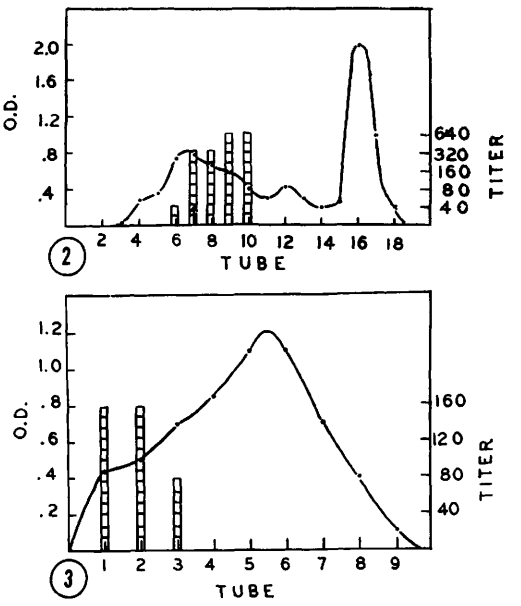

FIG. 2. Electrophoretic pattern obtained by zone electrophoresis in starch using barbital buffer pH 8.6 μ 0.05. Latex agglutination titer of each fraction is shown by the vertical bars.

FIG. 3. Density gradient centrifugation of a normal chicken serum. Vertical bars indicate latex agglutination titer of each fraction; O.D. represents protein concentration. Tube 1 sediments most rapidly.

each of the fractions making up the gamma and β globulins revealed a peak sedimenting ahead of the 19S component only in tubes 7-10 which also had the greatest agglutinating capacity. While a 19S peak was again present in the α_2 globulins, no other fraction had the agglutinating capacity or the component sedimenting ahead of the 19S peak. Attempts to calculate the $S^\circ_{20,w}$ of this component failed because of the small amounts of these proteins present in normal chicken sera as well as the presence of large amounts of more slowly sedimenting molecules in all preparations.

To determine more directly that the agglutinating activity was associated with the rapidly sedimenting proteins of high molecular weight, 6 sera were subjected to zone centrifugation in a density gradient. The distribution of protein and agglutinating activity of one such experiment is shown in Fig. 3. The results indicate that all of the agglutinating activity was associated with the 3 most rapidly sedimenting fractions containing less

than 10% of the protein which were recovered in aliquots corresponding to those which contained all of the 19S gamma globulins of a normal human serum separated in another tube during the same experiment. Agglutinating activity was never present in the more slowly sedimenting fractions which contained the bulk of the proteins.

Previous studies with human 19S gamma globulins and the 22S rheumatoid factor complex have shown that these proteins are held together by disulfide bonds which are readily dissociated by sulfhydryl compounds. Such dissociation is accompanied in all instances by complete loss of serologic activity(12). Treatment of chicken sera or gamma globulins with 0.1 M mercaptoethanol resulted in complete and irreversible loss of agglutinating activity. This was accompanied by disappearance of the high molecular weight peaks and increase in the more slowly sedimenting components. Urea or a pH of 3 resulted only in the disappearance of the more rapidly sedimenting peak leaving the 19S peak intact. These changes were accompanied by loss of serologic activity in presence of the reagents but almost complete recovery of activity following their removal.

Discussion. Makinodan, Gengozian and Canning(3) have recently described the presence in chicken sera of high molecular weight γ-globulins and have demonstrated their identity with those substances long known to coprecipitate with antigen antibody complexes(1,2). However, little is known about the biologic significance of these substances. The present report confirms and extends previous observations on the structure of these proteins, and presents a simple technic for their study based on ability of these proteins to agglutinate latex particles. As previously shown for other biologically active 19S gamma globulins in man and other species, agglutinating activity is associated only with the high molecular weight proteins in their native state and not with products obtained after depolymerization(12). While the agglutinating factor belongs to the same high molecular weight fraction as the proteins previously shown to coprecipitate(3), failure to absorb the agglutinating activity with antigen antibody precipitates suggests that a different protein belonging to the high molecular weight gamma globulins is responsible.

The nature of the interaction between the latex particles and other inert particles and the high molecular weight proteins in chicken sera is not clear. However, 2 possibilities arise; either the proteins may interact with the negatively charged latex particles directly, or they may react with gamma globulins known to coat these particles which are also present in antigen antibody complexes. At present it is not possible to choose conclusively between these 2 alternatives since latex particles may be readily coated with gamma globulins including homologous gamma globulin prior to reacting with the chicken factor. However, the inhibition of agglutination by addition of a number of proteins, especially those carrying a positive charge, including human and rabbit gamma globulins suggest that under the conditions of the study these may coat the particles preferentially and thus block their agglutination by the chicken proteins by neutralizing their negative charge.

Summary. 1. Agglutination of latex particles and other particulate bodies by chicken sera has been shown to be due to presence of certain high molecular weight gamma and β globulins. This technic promises to be a simple reliable tool to study some of the properties of these proteins. 2. It has been shown that these proteins are active only in their native state and not following depolymerization. 3. Agglutination is inhibited by prior addition of other proteins, especially those with a basic charge. This is probably due to prior coating of the particle by these proteins.

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A Spectrophotometric Method for Determination of Elastase and Serum Elastase Inhibitor.* (27196)

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The presence of an elastolytic enzyme in pancreatic extracts and the inhibiting effect of human and various animal sera on this enzyme were described by Balo and Banga(1, 2). Methods for the assay of both elastase and its serum inhibitor have included gravimetric(3), Kjeldahl(1), agar plate(4) and nephelometric(5) technics. Sachar *et al.*(6) measured with a spectrophotometric technic the amount of dye released into solution from orcein-impregnated elastin by enzymatic digestion. In attempting to adapt this method to measurement of the serum elastase inhibitor it was found that opalescent sera produced significant errors in the optical density readings. To obviate this the above method was modified by extracting the liberated orcein dye from solution with n-butyl alcohol prior to the spectrophotometric determination.

Materials. Substrate. Purified elastin-orcein powder.‡ Buffers: Primary standard grade Tris buffer [Tris (hydroxymethyl) aminomethane] 1/5 M, pH 8.8. Reagent grade phosphate buffer, 1/15 M, pH 6.0. Enzyme: Crystalline pancreatic elastase.§ A unit of activity as defined by Sachar *et al.* (6) is that amount of enzyme which will solubilize 1 mg of elastin-orcein in 20 minutes under the prescribed conditions. Each sample of enzyme is tested for activity and standardized accordingly. Acid for protein precipitation: Reagent grade 40% trichloroacetic acid (TCA). Extracting medium: Reagent grade n-butyl alcohol. Serum: Venous blood samples obtained prior to the morning meal from 17 patients on the Gynecology Service of the Sloane Hospital for Women, Columbia-Presbyterian Medical Center. The serum was harvested from clotted blood in the customary manner and immediately frozen. Samples were assayed for elastase inhibitor within 2 weeks from date of collection. Samples of sera which were stored at minus 20°C, at 4°C and at room temperature (22°C) showed no change in inhibitor activity at the end of 2 weeks. Hemolyzed sera were not used.

Method for measurement of elastase. Duplicate 25 mg samples of elastin-orcein are placed in 1.5 × 10 cm tissue culture tubes fitted with plastic lined screw caps. Two ml of Tris buffer are added to the substrate in each tube. A 1 ml suspension of elastase in Tris buffer containing 30 units of elastase activity per ml of suspension is added to the reaction mixture in each tube. The screw caps are secured on the tubes and the tubes

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