Unusual Sedimentation and Sulfhydryl Sensitivity of Certain Isohemagglutinins and Skin-Sensitizing Antibody.* (27437)

JOHN H. ROCKEY AND HENRY G. KUNKEL
The Rockefeller Institute, New York City

Studies from many laboratories have established the existence of two classes of antibodies differing in sedimentation characteristics and sensitivity to disulfide bond reducing compounds(1,2). Antibodies of one class have sedimentation rates of approximately 7S. Those of the second class have sedimentation rates of approximately 19S. The latter are relatively sensitive to disulfide bond reducing compounds, dissociating into smaller units and losing the capacity to combine with antigen upon such treatment.

The present study reports experimental evidence for the existence of a third class of antibodies with intermediate sedimentation rates. Analysis of certain isohemagglutinins by density gradient ultracentrifugation established that a large proportion of the antibodies had sedimentation rates in the range of 9-15S. A skin-sensitizing antibody reacting with highly purified mammalian glucagon was shown to have a sedimentation rate in the range of 8-11S. Both the agglutinating activity of the intermediately sedimenting isohemagglutinins and the skin-sensitizing capacity of the reaginic antibody were inactivated by treatment with 0.1M mercaptoethanol.

Method and materials. High titer anti-A and anti-B sera from Group B and A individuals, immunized with specific blood group substances, were used. These were obtained from various sources and included sera from the Knickerbocker Blood Bank and one serum kindly furnished by Dr. R. Rosenfield. Purified anti-A antibodies were prepared by elution from A stroma with 6M urea or acid buffer of pH 3.0. Eluates were dialyzed against buffered saline. Skin-sensitizing antibody reacting with highly purified glucagon was obtained from an allergic diabetic male patient of Drs. E. Bierman and E. Gordis.

Density gradient ultracentrifugation was accomplished as previously described(2) with minor modifications. Continuous gradients were formed by the dropwise addition of concentrated sucrose solutions of either 30 or 40% to 10% sucrose in a mixing chamber. Diluted serum samples of from 0.1-0.4 ml were layered on the gradients. Centrifugation was carried out in a Spinco Model L machine using a swinging bucket rotor (SW 39) at 35,000 rpm for periods ranging from 14-24 hours. Serial fractions were collected under controlled pressure through a standard needle perforating the bottom of the centrifuge tube. The sedimentation characteristics of the isohemagglutinins and of the skin-sensitizing antibody were determined by simultaneous sedimentation with established 7S and 19S antibodies. The titer of each activity was determined for each fraction. The distributions of 7S γ-globulin, 19S γ-globulin and β_{2A}-globulin in the fractions were determined by agar diffusion and capillary tube precipitation with specific, absorbed antisera. Agglutination reactions were performed in the usual manner in a phosphate-buffered saline solution of pH 8.0.

Passive transfer reactions were carried out in normal human skin. Sites were challenged with antigen injected 24-72 hours after sensitization. Purified crystalline glucagon (Lilly Lot 258-234B-167-1) containing less than 0.05% insulin by immunoassay(3), and approximately 0.01% insulin-like activity by epididymal fat pad assay(4) was used as antigen. Reactions were graded on a negative to 4+ scale. A reaction was graded 4+ when the area of induration was 0.75 cm or greater, and the area of erythema 3.0 cm or greater in diameter. Final readings were made at 30 minutes.

DEAE column chromatography(5,6) was accomplished by elution with a continuous salt gradient formed by addition of 1M NaCl in initial buffer to 0.02M phosphate buffer of pH 7.68. Distribution of agglutinating activity was determined and various fractions

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were pooled and concentrated by ultrafiltration.

Mercaptoethanol was added to serum or antibody preparation to a final concentration of 0.1M. Mixtures were incubated at room temperature for 4 hours and then dialyzed against either physiological saline or 0.02M iodoacetamide in saline. Controls were similarly treated excepting that mercaptoethanol was omitted.

Results. Density gradient ultracentrifugation. The use of an improved method of zone ultracentrifugation in a sucrose gradient indicated that certain high titer isohemagglutinin sera contained a type of antibody which differed from the classical 7S and 19S antibodies in sedimentation characteristics. Such antibodies were the dominant population in some sera. They were also found together with classical antibodies in various proportions in other sera.

Fig. 1 presents results of a typical experiment. Simultaneous ultracentrifugation of an established 19S antibody, cold hemagglutinin(7), and an established 7S antibody, incomplete anti-Rh antibody(7,8), permitted definition of the sedimentation characteristics of the unusual isohemagglutinins. The anti-A antibodies in this particular serum sedimented in a wide distribution with peak activity intermediate between those of the 7S and 19S antibodies. The distribution of 7S and 19S γ-globulins, determined immunologically with specific antisera, was similar to the distribution of the incomplete anti-D antibody and the cold hemagglutinin respectively. The B_{2A}-globulin distribution was similar to that of the 7S γ-globulin.

A similar experiment is represented in the upper portion of Fig. 2. This second serum contained significant amounts of both 19S and intermediately sedimenting antibody.

Eight high-titer anti-A sera from group B individuals were similarly studied. In 2 the intermediately sedimenting antibodies were in marked preponderance. In 2 they appeared to be absent, and antibodies of the 19S class were the dominant population. In the remaining sera mixtures of intermediately sedimenting and classical antibodies were found. Antibodies with intermediate sedimentation rates were also encountered in 2 high-titer anti-B sera.

Highly purified preparations of anti-A antibodies were prepared by elution from A stroma. Antibodies with intermediate sedimentation rates were found in eluates from sera containing significant amounts of such antibodies.

The sedimentation characteristics of the skin-sensitizing antibody were determined in a similar manner. The results of a typical density gradient ultracentrifugation experiment are presented in Fig. 3. Here, incomplete anti-D antibody was centrifuged simultaneously with the skin-sensitizing antibody. The skin-sensitizing activity sedimented more rapidly than did the anti-D antibody activity. The resolution obtained between the 4.5S albumin peak and the 7S incomplete anti-D antibody peak is consistent with a sedimentation rate of approximately 8-11S for the skin-sensitizing antibody. Density gradient ultracentrifugation of a fresh serum sample established that the skin-sensitizing activity sedimented in a manner similar to the hemolytic activity of the β_{1C} component of complement(9). β_{1C}-globulin has a sedimentation rate of $S^{\circ}_{20,w} = 9.5$(9). The possibility that distribution of skin-sensitizing activity was dependent on β_{1C} was considered. However, the skin-sensitizing activity could be separated from the β_{1C}-hemolytic activity by zone electrophoresis. In addition, antiserum which had been decomplemented by absorption with antigen-antibody complexes at 37°C retained skin-sensitizing capacity.

Centrifugation experiments designed to clearly separate the 19S macroglobulins from the remaining serum proteins in a zone above the bottom of the centrifuge tube failed to show any skin-sensitizing activity associated with these macroglobulins.

DEAE chromatography. Anti-A sera containing significant amounts of intermediately sedimenting antibodies were analyzed by gradient elution DEAE chromatography. The major agglutinating activity of such sera was eluted in a wide distribution after elution of the major 7S γ-globulin peak and usually commenced just after the principal albumin peak. With some sera, little if any activity

was associated with the principal 7S γ-globulin peak. Various fractions showing agglutinating activity were pooled and concentrated. The sedimentation characteristics of the contained antibodies were determined by density gradient ultracentrifugation. Fig. 2 presents the results of a typical experiment. Here, whole serum and pooled concentrated fractions from 2 successive regions of the chromatogram were sedimented simultaneously. Two activity peaks were obtained on ultracentrifugation of the whole serum. These were separated in the 2 successive fractions from the latter regions of the chromatogram.

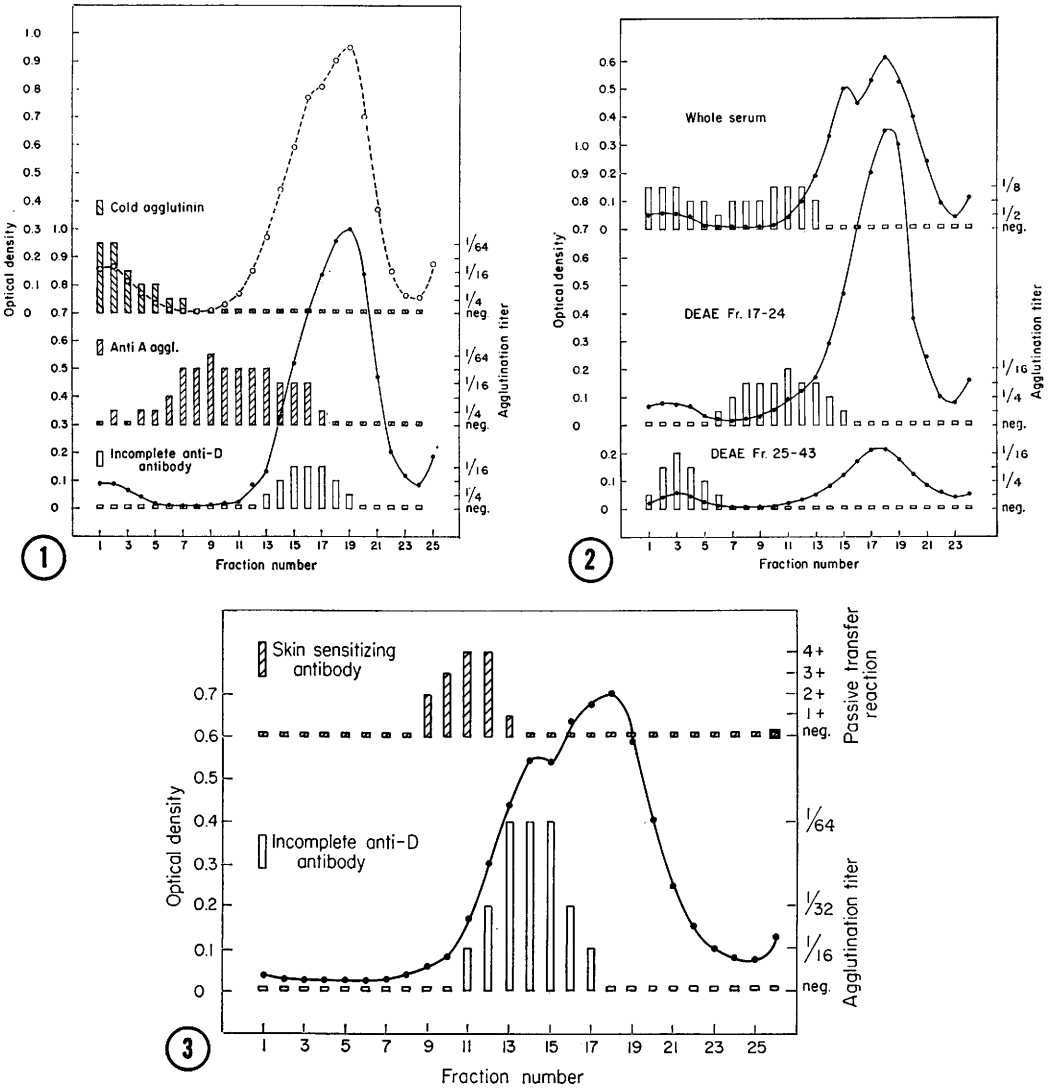

FIG. 1. Curves of protein concentration and distribution of agglutination obtained by simultaneous density gradient ultracentrifugation of anti-A 6460 isohemagglutinin, incomplete anti-D antibody and cold agglutinin. Base line values represent negative agglutination at lowest dilution indicated. ●——● Mixture of anti-A and anti-D antibodies. ○---○ Mixture of anti-A and cold hemagglutinin. Only the cold hemagglutinin activity is recorded.

FIG. 2. Simultaneous ultracentrifugation of whole anti-A serum 49 and of pooled concentrated fractions from the DEAE chromatogram of the same serum.

FIG. 3. Curve of protein concentration and distribution of skin-sensitizing activity and agglutination obtained by simultaneous density gradient ultracentrifugation of reaginic antiglucagon antibody and incomplete anti-D antibody.

TABLE I. Effect of Mercaptoethanol on Intermediately Sedimenting and Classical 7S and 19S Antibodies.

	Mercaptoethanol		Control	
	Saline	Iodoacetamide	Saline	Iodoacetamide
Intermed. abs.				
Sera 49	4	4	1024	512
" 39	< 2	< 2	1024	512
" 22	<20	<20	640	640
" 6460	64	16	1024	2048
Eluate 22	16	—	128	—
DEAE 6460	< 4	< 4	512	1024
19S antibodies				
Anti-A	2	< 2	1024	512
Cold agglutinin	<16	<16	4096	4096
Anti-D	1024	512	1024	512
7S 6460	32	32	32	32
Skin-sensitizing antibody	neg.	—	4+	—

Numerical values are reciprocals of agglutination titers. <, negative at lowest dilution.

The majority of the intermediately sedimenting antibodies were eluted before the 19S antibodies.

Mercaptoethanol treatment. The results of treatment of the various antibodies with 0.1M mercaptoethanol are presented in Table I. Mercaptoethanol markedly reduced the agglutinating titer of anti-A sera, stroma eluates and pooled concentrated DEAE column fractions containing large proportions of intermediately sedimenting antibodies. The agglutinating activity of the cold agglutinin and a 19S anti-A serum also were markedly reduced by such treatment. The capacity of the reaginic anti-glucagon serum to sensitize skin was inactivated by mercaptoethanol treatment† followed by dialysis against saline. In contrast to this, the titers of 7S anti-A and anti-D antibodies were essentially unchanged by such treatment.

It was established that skin sites injected with mercaptoethanol-treated serum were still able to respond by simultaneously injecting both treated and untreated antisera into normal skin. Such sites responded with a strongly positive reaction when subsequently challenged with antigen.

Other properties. Both the intermediately sedimenting isoagglutinins and the skin-sensitizing antibody migrated between the γ- and β_2-globulin peaks on zone electrophoresis(10, 11). The ability of the reaginic antibody to sensitize skin was inactivated by heating at 56°C for 2 hours. Similar treatment of the intermediately sedimenting isohemagglutinins did not change their agglutinating titers significantly.

Discussion. These experiments provide evidence for the existence of an unusual class of sulfhydryl-sensitive antibodies with sedimentation rates intermediate between those of the 7S and 19S class antibodies. Isohemagglutinins of the latter 2 classes have been identified previously(7), but methods have not been available to delineate clearly the intermediate type. It is probable that in the past such antibodies were included with the 19S group. Various antibodies have been classified on the basis of sulfhydryl sensitivity and DEAE column chromatography as either 7S or 19S γ-globulins. These results must be reinterpreted with the knowledge of the existence of intermediately sedimenting antibodies and of their behavior under such conditions.

The sedimentation characteristics of skin-sensitizing antibody have not been clearly defined by the methods previously employed. Sehon and coworkers have suggested that they may be 19S class antibodies and sedimentation rates ranging from 12.4 to 22.5S have been calculated from partition cell centrifugation data(12). Other workers have concluded that they are of the 7S class(13,14, 15). The various technics employed would not have clearly resolved intermediately sedimenting antibodies from 7S and 19S antibodies. Skin-sensitizing antibodies thus far examined by DEAE chromatography have been eluted after the principal 7S γ-globulin component(13,15).

The unusual components observed in some purified horse anti-pneumococcal antibody preparations(16) may be related to the antibodies described here.

A major question concerning the intermediately sedimenting antibodies is their antigenic nature. The possibility that they are related to the β_{2A} immunoglobulins(17) certainly re-

† These results confirm the unpublished observation of J. P. Leddy that skin-sensitizing antibody is inactivated by sulfhydryl compounds.

quires consideration. They were separated from the major 7S component of the β_{2A}-globulins in the ultracentrifugation experiments. However, certain β_{2A} myeloma proteins have sedimentation characteristics comparable with those of the intermediately sedimenting antibodies(18). Such myeloma proteins reduce to smaller units on treatment with disulfide bond reducing compounds(19, 20). It seems possible that the unusual antibodies are related to these myeloma components. Their exact antigenic characterization awaits their complete isolation which thus far has not been accomplished.

The unusual isohemagglutinins and skin-sensitizing antibodies are included together in the present report because of the similarity of their ultracentrifugation, DEAE chromatographic, electrophoretic, and sulfhydryl sensitivity properties. There is no evidence that they are otherwise related.

Summary. Improved methods of density gradient ultracentrifugation have demonstrated that, in addition to the well-known 7S and 19S class antibodies, antibodies with intermediate sedimentation rates exist. Analysis of certain isohemagglutinins in whole sera and purified fractions have shown that a large proportion of the antibodies have sedimentation rates in the range of 9-15S. These antibodies, clearly separable from 19S class proteins, were inactivated by treatment with 0.1M mercaptoethanol. A skin-sensitizing antibody reacting with highly purified glucagon had a sedimentation rate in the range of 8-11S. The ability of this reaginic antibody to sensitize skin as measured by the classic Prausnitz-Kustner reaction also was inactivated by treatment with 0.1M mercaptoethanol. The possible relationship of these antibodies to the β_{2A} class of immunoglobulins is discussed.

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