ever, in one case a reaction specific for hemoglobin F was obtained by the inter-facial ring test. The infants in each case showed clinical manifestations of hemolytic disease, some requiring replacement or supplementary transfusions. We have as yet failed to clarify the nature of this antibody, other than establishing its existence.

It was not possible to substitute guinea pig complement for human complement in the two-stage direct Coombs test contrary to our findings in the complement consumption studies in which the two were interchangeable.

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High Molecular Weight Antibodies

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It has long been known that normal human serum contains small amounts of a high molecular weight substance with sedimentation rate of about 19 Svedberg units. Simultaneous separation on the same starch block of a normal human serum and of the heavy molecular weight component of the same serum, obtained by re-peated cycling in the preparative ultracentrifuge, discloses that the heavy com-ponent is comprised of two peaks of different electrophoretic mobility, and also discloses that the 19S gamma-globulin has an electrophoretic mobility slightly faster than that of the bulk of the gamma-globulin. Recently it has become apparent that 5-10 per cent of the total gamma-globulin of normal serum consists of this 19S component (molecular weight around one million). The other 90–95 per cent consist of material with sedimentation of 7 Svedberg units, implying a molecular weight of approximately 160,000. Gamma-globulin intermediate in molecular

weight between the 7S and 19S components does not occur in significant amounts in normal human serum.

The 19S and 7S gamma-globulin also differ in chemical composition and in their behavior as antigens, as has been demonstrated by previous papers from this laboratory. Both components contain a number of antibodies or antibody-like substances. A method of sucrose density-gradient ultracentrifugation has been employed in this laboratory for separation and isolation of the 7S and 19S components of gamma-globulin, thus permitting a classification of antibodies and other serologically active proteins into the 7S and 19S groups.

The first antibody studied was the high-titer cold agglutinin present in the serum of certain patients with chronic hemolytic anemia and paroxysmal cold hemoglobinuria. Sera from ten patients were obtained for study. Titers varied
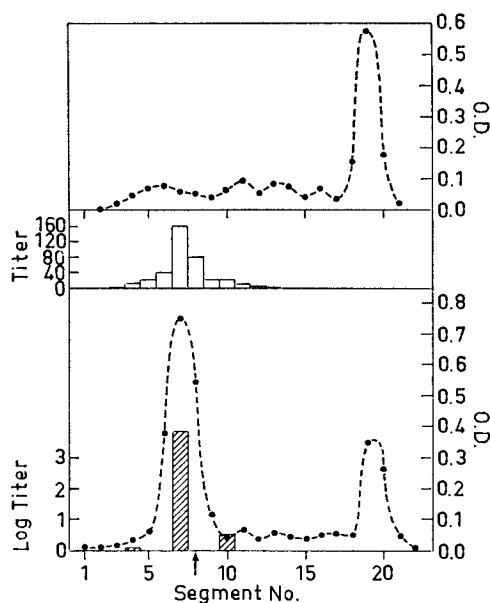

Fig. 1

from 1000 to 160,000. The bottom portion of fig. 1 represents the patterns obtained by protein analysis of the fractions obtained on starch block electrophoresis of the highest-titer serum. The agglutination titers (expressed in log units), obtained from each segment of the starch block are plotted immediately below the protein curve. More than 90 per cent of the total cold agglutinin activity was recovered in the various fractions; and it is evident that the localization of the cold agglutinin activity corresponds with that of the abnormal protein peak. The sedimentation pattern obtained on ultracentrifugal analysis of the starch block fraction containing the huge peak of cold agglutinin activity revealed a sedimentation
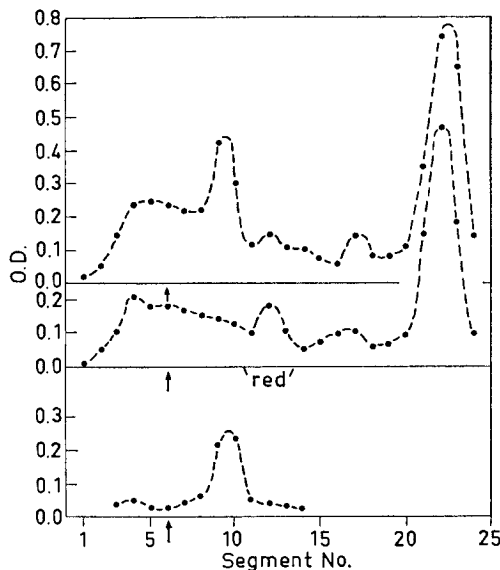
Fig. 2

coefficient, calculated to infinite dilution and corrected for ionic strength, of approximately 19 Svedberg units. No significant amount of 7S protein was present in the starch block fraction containing the cold agglutinins. The upper portion of fig. 2 demonstrates the electrophoretic pattern obtained with another cold agglutinin serum, titer 32,000. Although the site of the agglutinin activity is not plotted; it corresponded exactly with that of the abnormal protein peak. Following repeated absorptions of the serum with trypsin-treated red cell stromata in the cold, 95 per cent of the cold agglutinin activity was lost. The abnormal peak was also lost, as is evidenced by the middle portion of the slide. The position of the red hemoglobin added with the stromata is also indicated. The bottom portion of this slide is the electrophoretic pattern of the eluted cold agglutinin. The ultracentrifugal study of isolated cold agglutinin material, established its 19S character. Dr. *Dacie* and his co-workers have come to similar conclusions somewhat similar methods.

Use of a sucrose density-gradient method of zone ultracentrifugation permitted separation of 19S and 7S moieties of gamma-globulin, as indicated by fig. 3 depicting the plastic container after ultracentrifugation at 33,000 g for 18 hours.

The blue band represents albumin, localized by addition of bromphenol blue to the serum prior to layering on the sucrose gradient with a high titer cold agglutinin caused a clear increase in fraction (4) of the density gradient fractions. This fraction, reacting specifically with 19S antiserum, contained 90–95 per cent of the cold agglutinin activity. Fraction 2, containing a much higher protein concen-

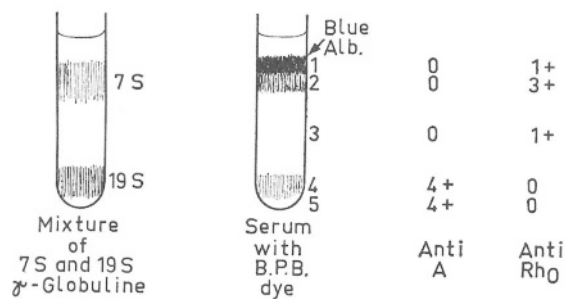|	Blue Alb.		Anti A	Anti Rh_0
7 S		1	0	1+
		2	0	3+
		3	0	1+
19 S		4	4+	0
		5	4+	0
Mixture of 7 S and 19 S γ-Globuline	Serum with B.P.B. dye			

Fig. 3

tration and the bulk of the 7S gamma-globulin as revealed by reactions with the anti-7S antiserum, was completely devoid of cold agglutinin activity. Similar results were obtained with other cold agglutinin sera with varying titers. Several sera of patients with warm antibody hemolytic anemia as manifested by strongly positive direct and indirect Coombs tests were studied. The warm incomplete antibody in these sera was found in the upper fractions with the peak of the 7S gamma-globulin; no activity was present in fractions 4 or 5 corresponding to the 19S gamma-globulin. Thus the antibodies of warm hemolytic anemia belong to the 7S component of gamma-globulin, the antibodies of cold hemolytic anemia to the antibodies of the 19S component.

With the density gradient method standardized with the cold agglutinin, attention was next centered on other serum proteins with serologic attributes. The L. E. factor, responsible for the induction of the morphologic changes in white cells comprising the L. E. phenomenon was shown to be restricted to the 7S component gamma-globulin while the heterophile agglutinin of infectious mononucleosis fell into the density gradient fraction corresponding to the 19S component. This technique also demonstrated that the serologic factor responsible for the positive serologic reactions in patients with rheumatoid arthritis is also a member of the 19S gamma-globulins, as demonstrated by the sensitized sheep cell, F. II reaction, and latex fixation and sensitized human Rh_0 cell tests for the rheumatoid factor.

Various antibodies against specific human red cell antigens were also studied in a similar fashion: As a general rule, "incomplete" antibodies – that is those active by Coombs test at 37° C proved to be of 7S character – for example, Kell, Cellano and Duffy, and the incomplete Rh_0 and rh' antibodies. In general, the bivalent (complete) saline active antibodies proved to be of 19S character – for example saline anti-Rh_0 and anti rh''; anti-M, and anti-N; and anti-P. It was possible to separate an incomplete anti-Rh_0 and a saline-active anti-Rh_0 in the same serum into the 7S and 19S fractions respectively, so that they were now free of one another. In general, in keeping with the observation that the 19S gamma-globulins are in the fast gamma region, the above cited saline antibodies migrated more rapidly than did the incomplete (Coombs) antibodies. This point is well illustrated by fig. 4, showing the electrophoretic mobilities of an incomplete anti-
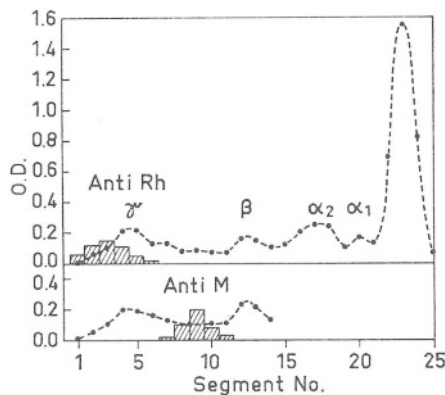
Fig. 4

Rh_O and a saline anti-M separated on the same starch block. "Incomplete" antibodies Kell, Cellano, and anti-rh' migrated in the slow or mid-gamma region, saline antibodies P, anti-Rh_O and anti-rh" in the fast gamma region.

One important exception to the general rule that incomplete, i. e. Coombs-reactive antibodies are 7 S and saline active antibodies 19 S was encountered during experiments performed with the anti-A iso-agglutinin of group O Rh-positive subjects. In a group of 12 normal subjects, three distinct patterns of saline anti-A activity were observed, as is evident from table I. In this table density gradient

Table I

Saline Anti-A Iso-Agglutinin Fractionation Patterns

Fraction Titer:	Type 1 (19 S pattern)					Type 2 (7 S pattern)					Type 3 (mixed)				
	5	10	20	40	80	5	10	20	40	80	5	10	20	40	80
I	0	0	0	0	0	++	+	0	0	0	++	+	0	0	0
II*	±	0	0	0	0	++++	+++	+	+	0	++++	+++	+	+	0
III	+	0	0	0	0	++	±	0	0	0	++	+	0	0	0
IV*	++++	+++	++	+	±	0	0	0	0	0	++++	++	+	+	0
V	+	±	0	0	0	0	0	0	0	0	++	+	0	0	0

fraction I corresponds to albumin, fraction II to 7 S protein and fraction IV to the 19 S gamma-globulin. It is evident that in some subjects only 19 S saline-anti-A activity was present as illustrated by the results in the left of the slide. In another group only 7 S saline anti-A was present (middle of slide) and in some, both the 7 S and 19 S fractions contained saline anti-A activity. Approximately ½ of the small group of normal males had a 19 S pattern, $1/6$th a 7 S pattern and $1/3$rd a mixed pattern – i. e. both 7 S and 19 S material.

Similar results were observed in a smaller group of normal type B male subjects with anti-A. Since less than ½ of normal subjects possessed 7 S saline

anti-A; either alone or in combination with 19S anti-A, the possibility that the anti-A pattern of group O mothers might be important in predisposing to ABO hemolytic disease of the newborn seemed to merit investigation, for a 7S antibody can traverse the placenta far more easily than can the large 19S molecule. We have thus far been able to obtain only 7 sera from O mothers of infants with ABO hemolytic disease. Although the small size of the group precludes definite conclusions, it is of interest that all 7 had 7S saline anti-A agglutinins, 3 7S alone, the 4 others 7S in conjunction with 19S saline anti-A. "Coombs-active" anti-A was present in the 7S fraction of these mothers. Investigations are being extended to see if resistance to Witebsky substance neutralization, acacia enhancement, and hemolysin activity cited by others as characteristic of the immune anti-A of ABO hemolytic disease are merely serologic attributes restricted to the 7S variety of iso-agglutinins.

The 19S antibodies like 19S proteins in general (10) also proved very sensitive to sulfhydryl reagents such as $1/_{10}$th molar mercaptoethanol; such treatment produced dissociation into smaller units with marked loss of agglutinin activity. This loss of serologic activity cited in a previous publication for the cold agglutinins (5) was also observed with the heterophile antibody and the rheumatoidc factor. Experiments with warm hemolytic anemia antibodies and incomplete Rh_0 antibodies demonstrated little or no loss of activity under similar experimental conditions. The 19S saline anti-A iso-agglutinin activity was usually partly destroyed but sera with 7S saline anti-A iso-agglutinins did not show significant reduction in activity when exposed to sulfhydryl reagents. This limitations of the sulfhydryl dissociation procedure as currently under study.

The presumed site of action of the mercaptoethanol is the disulfide bonds bridging the units comprising the 19S gamma-globulin. These bonds are disrupted upon the addition of mercaptoethanol or other sulfhydryl reagents breaking up the 19S gamma-globulin into subunits. Differences in antigenic composition of the 19S and 7S gamma-globulins (4), also exist. Though both components have one or more antigens in common, each possesses at least one antigen absent in the other; as demonstrated by precipitin curves and agar diffusion studies. Differences in chemical composition are also present between 7S and 19S materials. The subunits obtained following dissociation of the 19S gamma-globulin have qualitative differences in carbohydrate content, with approximately 5 times as much hexose sugar and sialic acid per mg peptide (2, 3).