

IMMUNOLOGICAL STUDIES OF THE 11S PROTEIN COMPONENT  
OF THE HUMAN COMPLEMENT SYSTEM\*

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The 11S protein,<sup>1</sup> or C'O, a component of fresh normal human serum, was first described by Müller-Eberhard and Kunkel (2) and Taranta *et al.* (3). It has been shown to have the following characteristics: heat lability, a sedimentation constant of 11, and the ability to combine with immune precipitates, aggregated gamma globulin, and sensitized sheep erythrocytes even in the presence of Na<sub>2</sub> ethylenediaminetetraacetate (Na<sub>2</sub> EDTA). Its heat lability and reactivity in the presence of Na<sub>2</sub> EDTA distinguished it from rheumatoid factors and from the classical first component of complement, respectively. Partial purification of the 11S protein by Müller-Eberhard yielded material which reprecipitated with soluble gamma globulin aggregates and reacted with sensitized sheep erythrocytes to form an intermediate complex in immune hemolysis, EAC'11S (4).<sup>2</sup>

Thus far the production of antisera to the 11S protein has not been described. The development of such antisera would provide a useful means for determining some of the characteristics and biological activities of this protein.

Recent studies have demonstrated that the cryoproteins formed in the sera of certain patients with systemic lupus erythematosus (SLE) required the presence of a heat-labile factor (5). Moreover, the formation of the cryoprecipitates was not inhibited by Na<sub>2</sub> EDTA, and purified 11S material reconstituted the precipitating property of heated SLE sera. These findings suggested that the 11S protein participated in cryoprecipitation.

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<sup>1</sup> Recently, it has been proposed to call the 11S protein C'1q (1); however, the previous nomenclature will be used in this paper.

<sup>2</sup> Standard terminology in complement experiments: EA, sensitized sheep erythrocytes, and C', complement.

In this study, rabbits were immunized with individual cryoproteins derived from SLE sera. The antisera produced reacted with partially purified 11S preparations as well as with fresh human serum and euglobulin preparations. Experiments, utilizing these antisera, have provided further evidence of the participation of the 11S protein in various immunological reactions of human serum. Of particular interest was the agglutination of EAC'11S complexes by these antisera thereby providing additional support for the concept that the 11S protein plays a critical role in human immune hemolysis.

### *Materials and Methods*

#### *I. Preparations of Human Serum.—*

*Human serum:* Fresh sterile human serum from single donors obtained commercially from Knickerbocker Biologics, Division of Charles Pfizer and Company, Inc., New York was used in most instances. Heating of serum and other preparations was done for 30 minutes at 56°C.

*11S protein preparations:* The 11S protein was partially purified according to the method of Müller-Eberhard and Kunkel (2) with these slight variations. Maximal yields were obtained if precipitates, formed in 0.01 M Na<sub>2</sub> EDTA between heat-aggregated human gamma globulin 8 to 10 cc, 30 to 50 mg/ml, and fresh human serum (200 cc), were dissolved in acetate buffer pH 4.5  $\mu$ 0.3 instead of the higher pH 5.4. Examination of representative 11S preparations in the Spinco model E ultracentrifuge showed less than 5 per cent contamination with 7 and 19S gamma globulins.

*R11S preparations:* Preparations lacking the 11S material (R11S) were prepared by repeated absorptions of fresh human serum with soluble human gamma globulin aggregates in 0.01 M Na<sub>2</sub> EDTA until rendered non-hemolytic. Following absorption, the preparations were centrifuged at 40,000 RPM in a 40.3 Spinco rotor for 90 minutes and aliquots of the supernatant stored in a dry ice chest. Usually 0.04 cc of the 11S preparations, when added to 0.1 cc R11S, restored complete lysis of sensitized sheep erythrocytes in 15 minutes.

*Euglobin and pseudoglobulin preparations* were obtained by dialyzing human serum against phosphate buffer pH 5.4,  $\mu$  0.02. The euglobulin precipitate was washed three times before dissolving in either Na<sub>2</sub> EDTA- or Ca<sup>++</sup>Mg<sup>++</sup>-containing buffers. Prior to dialysis some sera were incubated with 0.011 M hydrazine at 37°C for 45 minutes to obtain a reagent lacking C'4 (R4 serum) (6).

#### *II. Protein Separation Procedures.—*

*Zone electrophoresis:* Electrophoresis on polyvinyl chloride (pevikon) block was carried out as described by Kunkel (7) using phosphate buffer pH 7,  $\mu$  0.01. The protein concentration of eluates was estimated by the Folin-Ciocalteu technique (8).

*Immunelectrophoresis:* A modification of Scheidegger's technique (9) for agar gel immunoelectrophoresis was used. 0.7 per cent agar in a phosphate buffer pH 7.4  $\mu$  0.05 was poured on either 3 $\frac{3}{4}$  by 4 inch lantern slides or microscope slides. A potential of 20 to 25 v was applied for 3 hours with small slides and for 5 hours with the lantern slides. After immunodiffusion, slides were usually photographed prior to washing and subsequent staining with amidoblack.

*Density gradient ultracentrifugation:* 0.5 cc of various preparations were layered on a continuous sucrose density gradient from 10 to 40 per cent sucrose containing 0.01 M Na<sub>2</sub> EDTA and allowed to sediment in a Spinco model L ultracentrifuge at 35,000 RPM for 15 to 18 hours (10). Effluent fractions were obtained from a pinhole in the bottom of the tube.

*Ouchterlony plates:* Veronal buffer pH 7.6 with 0.7 per cent agar and 0.33 M glycine proved satisfactory for Ouchterlony plate analyses.

III. *Hemolysis*.—Sheep red blood cells were obtained under sterile conditions and stored in Alsever's solution. A 2-year-old lot of glycerinated rabbit anti-sheep erythrocyte serum (Cappel Laboratories, Westchester, Pennsylvania) stored at 4°C was used for amboceptor. The cells were sensitized with 4 hemolytic units (EA) according to the method of Kabat and Mayer (6). When Na<sub>2</sub> EDTA-sensitized cells were needed (11), the red blood cells were washed and sensitized in phosphate-buffered saline containing 0.01 M Na<sub>2</sub> EDTA and then rewashed twice in the same buffer. Most hemolytic titrations consisted of incubations for 30 to 60 minutes at 37°C using 0.2 cc of the sensitized cells in a total volume of 0.8 cc. The degree of lysis was expressed as per cent of complete hemolysis.

IV. *Agglutination Studies*.—The antisera were diluted serially in twofold dilutions and incubated at 4°C overnight in 0.01 M Na<sub>2</sub> EDTA containing buffered saline (pH 8.0). The agglutinations were read in the gross after the tubes had been centrifuged. Each tube contained 0.5 cc of diluted antiserum and 0.5 cc of a 0.5 per cent cell suspension. Two types of erythrocytes coated with antibody and 11S material were used. The intermediate EAC'11S was made according to the method of Müller-Eberhard (4) with rabbit hemolysin-sensitized sheep erythrocytes. A complement-binding human Rh system (12) was also used (D + human erythrocytes coated with an anti-D antibody known as Ripley). Equal volumes of Ripley serum diluted 1:32 and 1 per cent suspension of RH-positive cells were incubated for 45 minutes at 37°C in the presence of 0.01 M Na<sub>2</sub> EDTA. The sensitized cells were washed three times in saline and brought to a 1 per cent cell suspension. An aliquot of the sensitized cells was incubated with purified 11S for 3 hours at 4°C in 0.01 M Na<sub>2</sub> EDTA. These cells were again washed three times and resuspended to 0.5 per cent.

V. *Antisera*.—Rabbits were immunized with two types of antigens in complete Freund's adjuvant:<sup>3</sup> (a) cryoproteins from SLE sera, and (b) human C' absorbed to rabbit immune precipitates. Cryoprotein-containing sera from patients with SLE were allowed to stand for 48 hours at 4°C. The cold precipitates after being washed five times with buffered saline (pH 8.0) were emulsified with adjuvant and injected subcutaneously at weekly intervals for 6 weeks. Anticomplement antisera were made by the injection of individual rabbits with their own immune precipitates which had been formed in the presence of human complement. After 8 weekly injections with ovalbumen<sup>4</sup> in adjuvant, the rabbits were bled and quantitative precipitin analyses carried out. To 15 ml of the individually heated (56°C for 30 minutes) antisera, 50 ml of fresh whole human serum and ovalbumen sufficient to accomplish equivalence was added. The precipitates, which formed after 24 hours at 4°C, were washed five times with 50 ml cold saline and suspended in 20 ml of sterile isotonic saline. The rabbits were injected with their own immune precipitates (1 ml of equal volume of precipitate and adjuvant in four subcutaneous sites) at weekly intervals for 4 weeks. One week after the last injection, the animals were bled and the separated antiserum stored at 4°C. Many experiments were performed with anticryoprotein and anticomplement antisera which had been absorbed with an R11S reagent. The R11S reagent used for absorption was first heated to 56°C for 30 minutes and then lyophilized.

VI. Quantitative precipitin curves were performed according to Kabat and Mayer (6).

## RESULTS

A single component of fresh normal human serum could be demonstrated in gel diffusion studies if the four anticryoprotein antisera were absorbed first with a heated R11S reagent. Fig. 1 shows the results obtained when the anti-

<sup>3</sup> Difco Laboratories, Inc., Detroit.

<sup>4</sup> Nutritional Biochemicals Corp., Cleveland

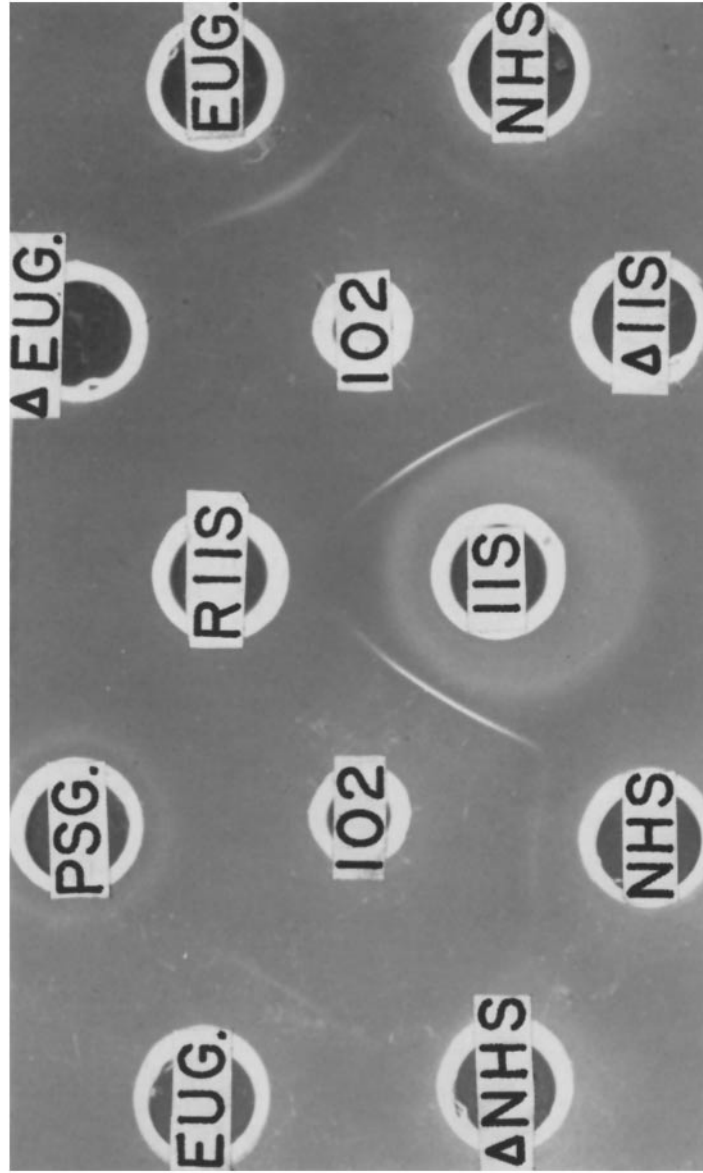


FIG. 1. Ouchterlony plate illustrating the recognition of a heat-labile antigen in various human serum preparations by the anticytoprotein antiserum 102. Both central wells contain antiserum 102 which had been absorbed with a heated lyophilized R11S reagent. Peripheral antigen wells contain normal human serum (NHS), heated serum ( $\Delta$  NHS), euglobin (EUG.), pseudoglobulin (PSG.), heated euglobulin ( $\Delta$  EUG.), "purified" IIS material (IIS), heated IIS ( $\Delta$  IIS), and an R11S reagent.

cryoprotein antiserum 102, absorbed with heated lyophilized R11S reagent, was allowed to diffuse against various preparations of human serum. Precipitin reactions occurred with fresh human serum, and with partially purified 11S and euglobulin preparations but could not be demonstrated in heated serum, R11S or pseudoglobulin preparations. Anticomplement antisera absorbed with an R11S reagent also demonstrated this same antigen in the same preparations.

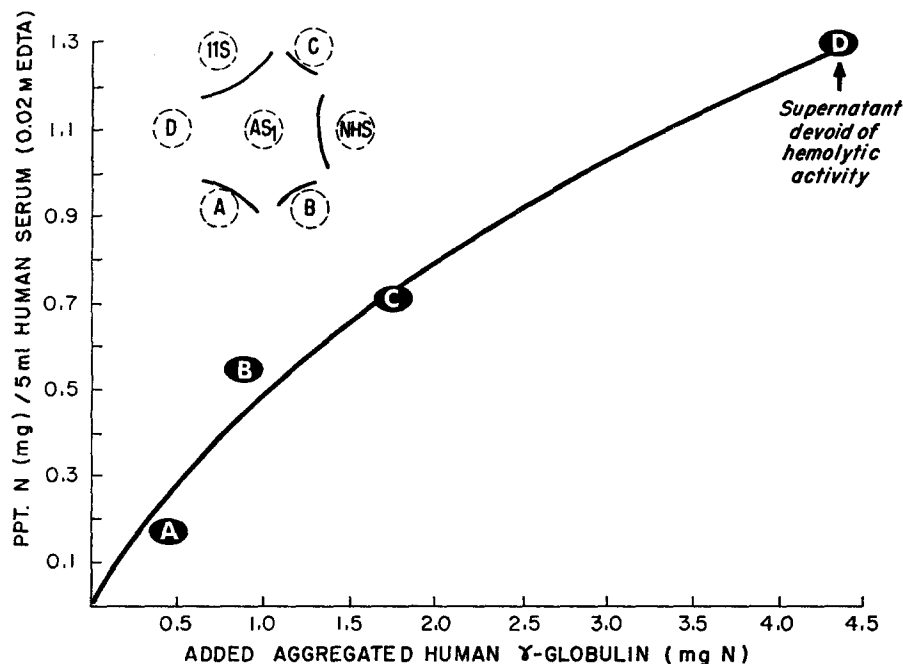


FIG. 2. Precipitin curve obtained by the addition of increasing amounts of soluble human gamma globulin aggregates to 5 ml aliquots of fresh human serum in 0.01 M  $\text{Na}_2\text{EDTA}$ . Ouchterlony plate drawing depicts the results obtained by diffusion of the supernatants of points A through D (outer wells) against the heated R11S-absorbed anticryoprotein antiserum 102 (center well).

Since this antigen was present in purified 11S preparations and absent in the R11S reagent, it was necessary to correlate its immunologic disappearance from the supernatants obtained by adding increasing amounts of human aggregated gamma globulin to fresh human serum performed in the presence of 0.01 M  $\text{Na}_2\text{EDTA}$ . Fig. 2 shows such an experiment. Supernatants of points A through D, in addition to normal human serum and purified 11S protein, were allowed to diffuse against an anticryoprotein antiserum. No precipitating antigen could be detected at point D suggesting that it had been completely removed by the aggregated gamma globulin. These supernatants were also centrifuged at

40,000 RPM for 90 minutes to remove soluble gamma globulin aggregates. The supernatant of point *D* showed no hemolytic activity despite restoration of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ .

The next series of experiments was designed to characterize this protein by the methods of protein separation available, correlating the immunoprecipitin analyses with hemolytic activity. Studies utilizing sucrose density gradient ultracentrifugation are summarized in Fig. 3. Three simultaneous gradients containing 0.5 cc normal human serum, 0.3 cc partially "purified" 11S protein, and 0.5 cc R4 euglobulin are illustrated. The whole R4 euglobulin preparation was obtained from 100 cc human serum and solubilized in 5 cc of 0.01 M  $\text{Na}_2$  EDTA-buffered saline. Hemolytic titrations utilizing 0.1 cc of an R11S reagent with 0.05 cc aliquots of the 11S and R4 euglobulin gradient fractions showed good correlation with the 11S protein peak. Furthermore, the heat-labile precipitin line produced by the anticryoprotein antisera in gel diffusion studies was localized to the same region as 11S hemolytic activity in the R4 euglobulin.

The same correlation between immunologic titrations with the anticryoprotein antisera and hemolytic titrations using an R11S reagent was also obtained on zone electrophoresis (Figs. 4 and 5). Here 11S hemolytic activity was demonstrated both by direct hemolytic titrations using 0.2 cc of the various block fractions with 0.1 cc of an R11S reagent and by forming the lysable intermediate complex EAC'11S. In this experiment the intermediate was obtained by incubating 1.0 cc aliquots with 0.2 cc EA for 10 minutes at 37°C and washing three times before adding the R11S reagent (Fig. 5). Forming the intermediate required at least five times as much 11S as was required for lysis when R11S and 11S reagents were added simultaneously to sensitized cells. Both the anticryoprotein and anticomplement antisera recognized the same heat-labile antigen in the gamma globulin region where the 11S hemolytic activity was localized.

Immunoelectrophoretic analyses of similar preparations of human serum are shown in Figs. 6 *a* and 6 *b*. The heat-labile 11S arc appeared in the mid-gamma globulin region distinct from the  $\gamma_2$ -globulin line. The same preparations that demonstrated this antigen by Ouchterlony technique formed precipitin arcs in immunoelectrophoretic studies except for normal human serum. The concentration of the 11S protein in serum was probably not sufficient for detection with these antisera. Furthermore, several commercial antisera to human sera did not appear to recognize the 11S protein.

Since the specificity of anticomplement antisera can be tested by the agglutination of specific complement intermediates or by interference with immune hemolysis, the former method was utilized to ensure that these antisera were, indeed, anti-11S. Table I illustrates agglutination studies obtained with a complement-binding anti-Rh system. Human D + erythrocytes coated with the Ripley anti-D serum and subsequently reacted with purified 11S protein were

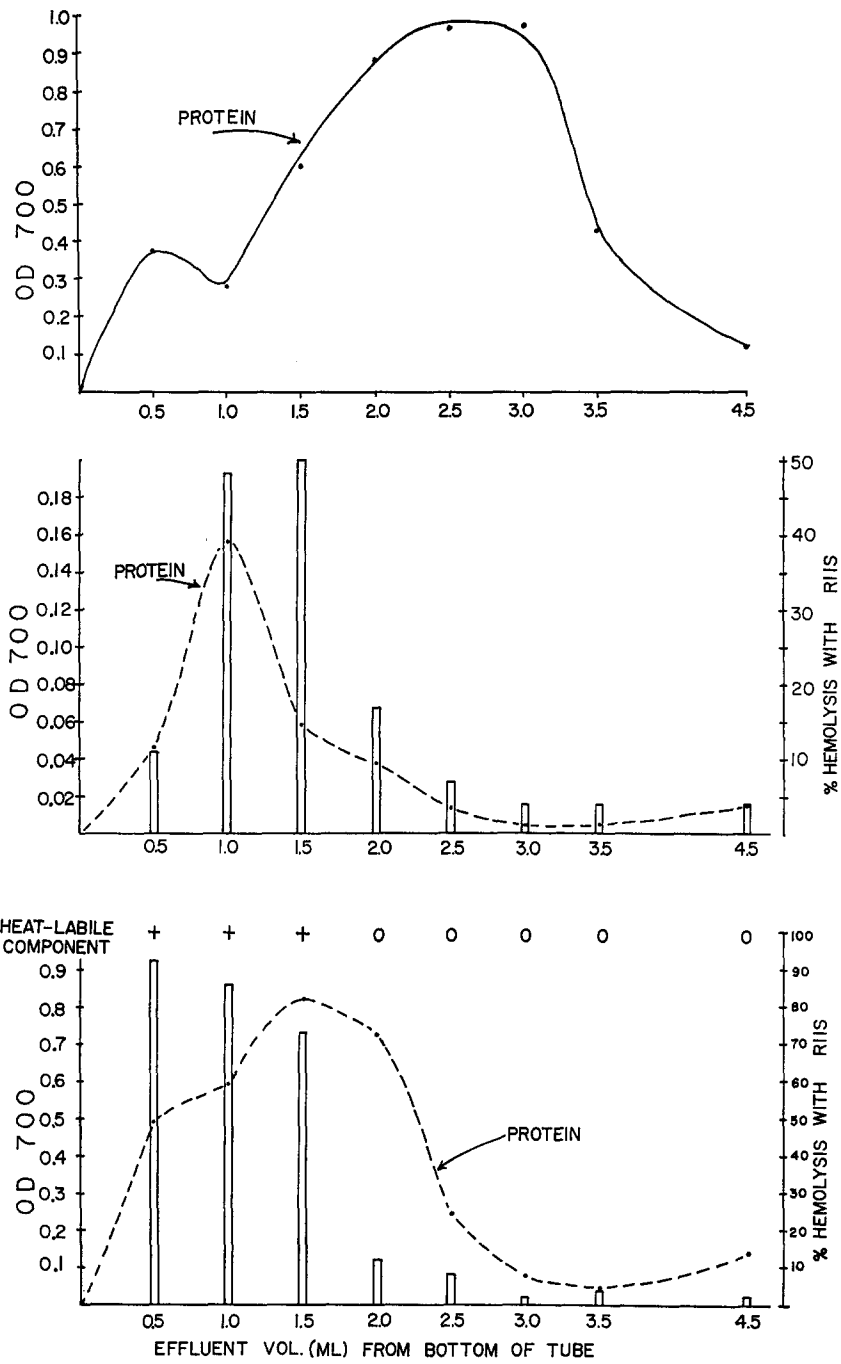


FIG. 3. Simultaneous sucrose density gradient ultracentrifugation experiments with normal human serum (top), "purified" 11S material (middle), and human R4 euglobulin (bottom). 0.5 cc fractions containing 0.01 M  $\text{Na}_2\text{EDTA}$  were collected from the bottom of the tube (left) to the top (right). The presence of the heat-labile line was determined by the anticryoprotein antiserum 102 in gel double diffusion studies.

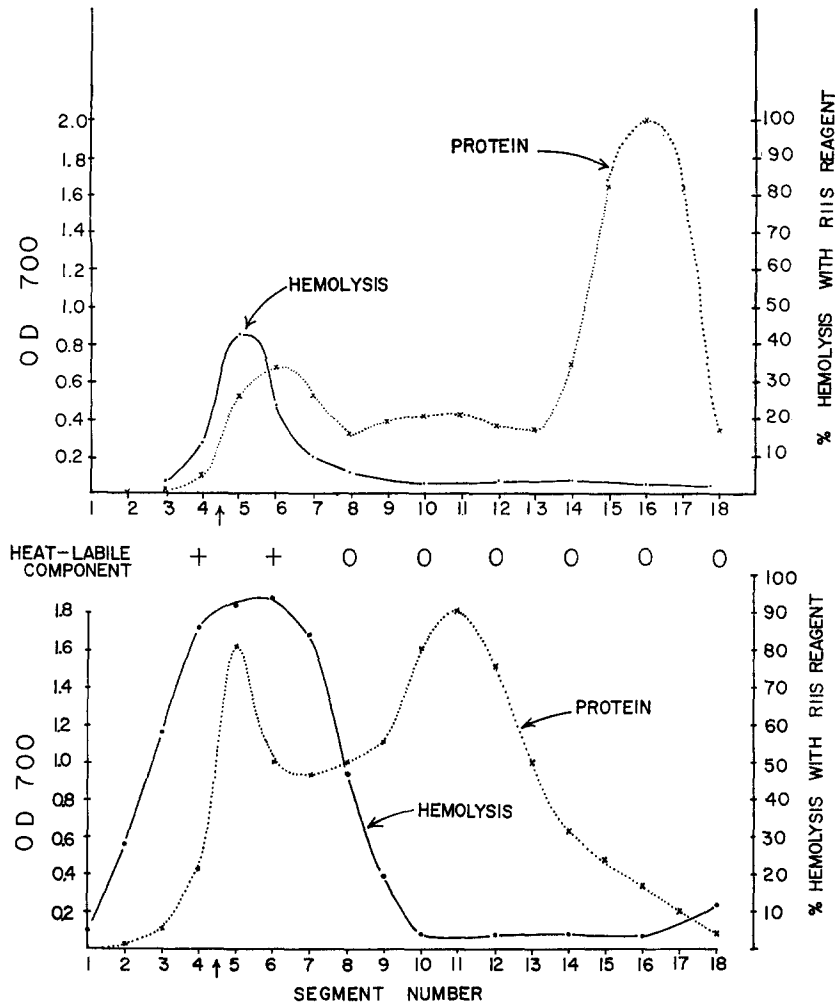


FIG. 4. Zone electrophoretic separation on pevikon of 1.5 cc human serum (top) and an R4 euglobulin preparation from 100 cc human serum which had been solubilized in 0.01 M Na<sub>2</sub> EDTA phosphate buffer (bottom). Antiserum 102 was again utilized to detect the presence of the heat-labile antigen.

agglutinated by the human gamma globulin absorbed anticryoprotein antiserum to a higher titer than the incomplete anti-Rh coated cells alone.

The agglutination of EAC'11S by anticryoprotein antisera was also studied using sheep erythrocytes sensitized with rabbit hemolysin (EA). In this particular experiment, an R4 euglobulin was incubated for 10 minutes at 37°C with EA and washed three times in buffered saline containing 0.1 M Na<sub>2</sub> EDTA.



Table II compares the agglutination of EA and EAC'11S by anticryoprotein antisera 102 and 83 which had been absorbed with human gamma globulin. An 0.5 per cent cell suspension provided optimum reading for agglutination studies whereas  $5 \times 10^8$  cells were used for hemolytic experiments. The EDTA-washed intermediate EAC'11S was lysed by the R11S reagent and agglutinated by the absorbed anticryoprotein antisera.

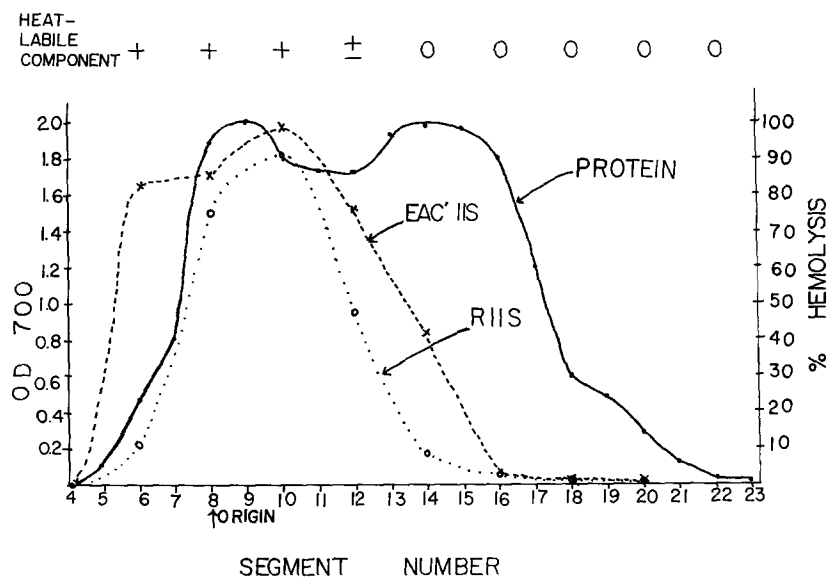


FIG. 5. Zone electrophoresis of a R4 euglobulin from 100 cc human serum solubilized in 0.01 M  $\text{Na}_2$  EDTA phosphate buffer (pH 7.0). The fractions in the gamma globulin region could be lysed in the presence of R11S or by first forming the intermediate EAC'11S before adding the R11S reagent. Anticryoprotein antiserum 102 absorbed with heated R11S reagent localized the heat-labile antigen to the same gamma globulin region.

#### DISCUSSION

The observation that the 11S protein was included in the cryoprecipitates formed from sera of patients with SLE provided a new source of antigen for the production of anti-11S antibodies. Absorption of these anticryoprotein antisera with lyophilized heated R11S reagent rendered them specific for a heat-labile antigen found in fresh human serum, purified 11S protein and euglobulin preparations and absent in heated serum, R11S, and pseudoglobulin reagents. Anticomplement antisera also recognized this antigen under the same conditions.

Further confirmation that these antisera, if absorbed with heated R11S, were specific for the 11S component, was obtained by comparing hemolytic titrations using the R11S reagent with immunologic titrations using these antisera. Good

correspondence between the two methods was found in both density ultracentrifugation and zone electrophoresis experiments. In fact, pevikon block fractions in the gamma globulin region could provide a source of 11S for forming the intermediate EAC'11S. The washed intermediate EAC'11S formed from

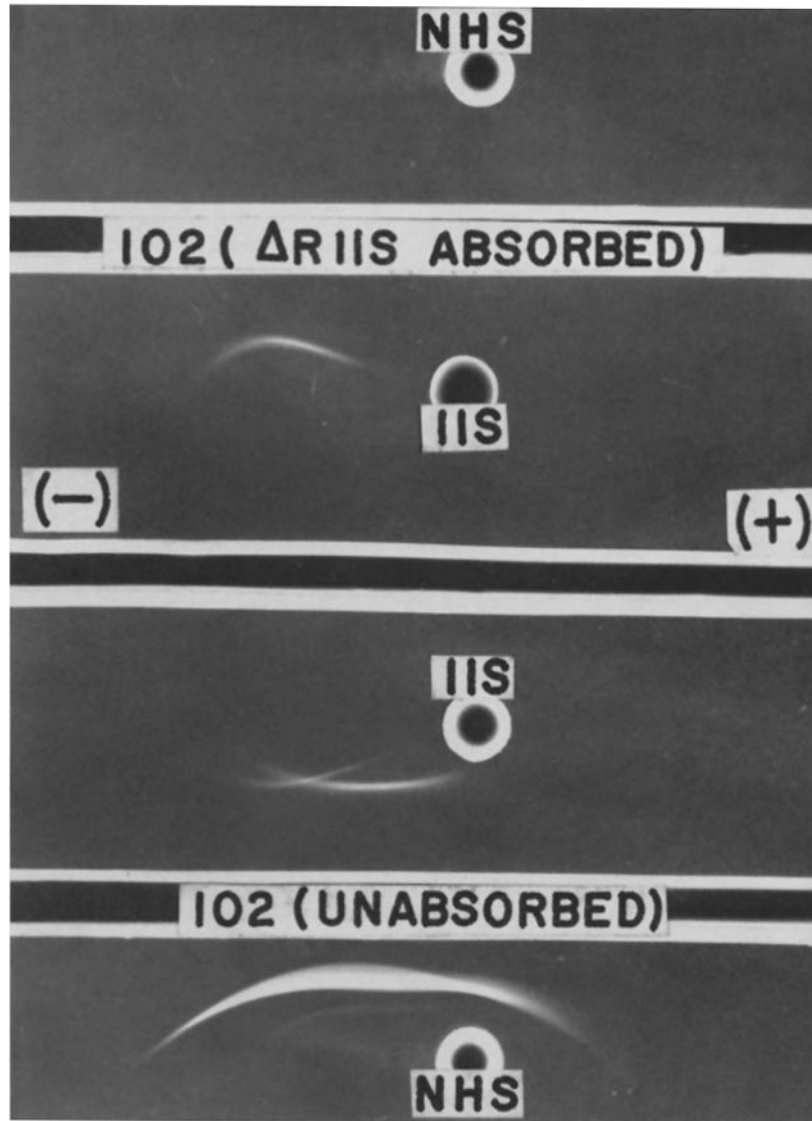


FIG. 6 a. Immunoelectrophoresis of normal human serum (upper and lower wells) and the 11S protein ( middle wells), developed with the heated R11S-absorbed anticytoprotein anti-serum 102 (upper trough) and unabsorbed antiserum 102 (lower trough).

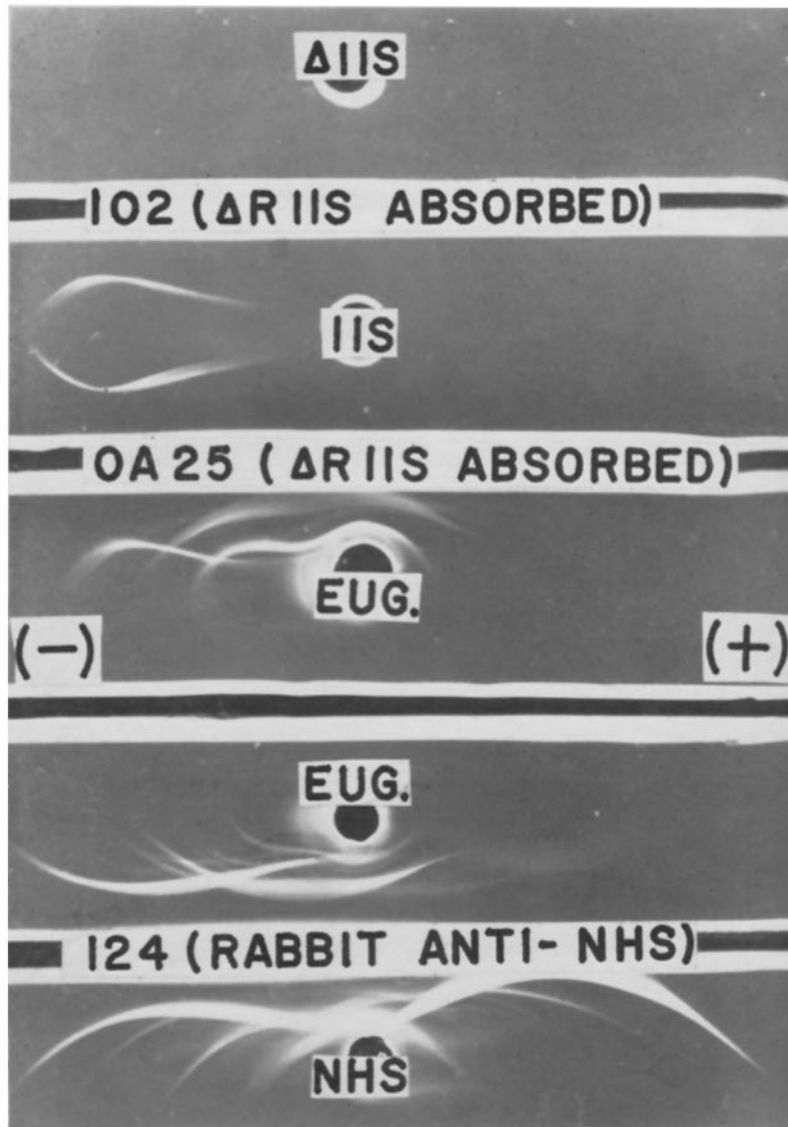


FIG. 6 *b*. Immunoelectrophoresis of heated 11S protein, unheated 11S protein, a euglobulin preparation, and normal human serum (wells from top to bottom). Troughs from top to bottom contained anticytoprotein antiserum 102 absorbed with heated R11S reagent, anticomplement antiserum OA 25 absorbed with heated R11S reagent, and an unabsorbed rabbit anti-human serum 124.

an R4 euglobulin in Na<sub>2</sub> EDTA could be lysed by the R11S reagent and agglutinated by the absorbed anticryoprotein antisera. These antisera also agglutinated the intermediate obtained from a completely human complement-

TABLE I  
*Agglutination Studies Comparing the Agglutination of Ripley-Sensitized Rh-Positive Erythrocytes Alone and with the Same Cells Which Had Been Incubated with the Purified 11S Protein*

	Dilutions of anti-SLE cryoprotein antiserum							
	20	40	80	160	320	640	1280	Titer
Cells incubated with 11S material (0.02 M EDTA).....	4+	3+	2+	2+	1+	1+	1+	1280
Cells incubated with saline (0.02 M EDTA).....	2+	1+	1+	1+	0	0	0	160

Human gamma globulin absorbed-anticryoprotein antiserum 83 was used as the antiserum

TABLE II  
*Agglutination Studies Comparing the Agglutination of EAC'11S and EA with the Human Gamma Globulin-Absorbed Antisera 102 and 83*

		Hemagglutination										Hemolysis		
		Dilutions of anti-SLE cryoprotein antisera											Saline cell control	Lysis with R11S reagent
		20	40	80	160	320	640	1280	5120	10,240	20,480			
Antiserum 83	EA	0	0	0	0	0	0	0	0	0	0	0	0	6
	EAC'11S	3	3	3	3	3	3	3	2	1	1	0	96	
Antiserum 102	EA	0	0	0	0	0	0	0	0	0	0	0	6	
	EAC'11S	3	3	2	2	2	2	2	1	0	—	1	96	

The source of 11S here was an R4 euglobulin prepared from 200 cc human serum which was also absorbed for 10 minutes at 4°C with EDTA-washed cells to remove the heterophile antibody. The formation and washing of the intermediate EAC'11S and the hemagglutination experiments were performed in 0.01 M Na<sub>2</sub> EDTA-buffered saline.

binding system. In this instance, the intermediate was formed by washing Ripley-coated RH-positive erythrocytes which had been incubated with a preparation of purified 11S.

As previously mentioned in the introduction, 11S protein antibodies have not previously been reported. Whether the 11S protein must be bound to immune complexes to be antigenic or whether the proper conditions for its

detection by immunologic means were fortuitously used cannot be stated. The immunization of rabbits with purified 11S preparations might help to answer this question. Although rigorous studies regarding pH and agar concentration were not conducted, the 11S protein could not be demonstrated in immunoelectrophoresis using veronal buffer pH 8.6  $\mu$  0.05 and agar concentrations between 1 and 2 per cent. Na<sub>2</sub> EDTA was not incorporated in the gel or zone electrophoresis media, but it was used in the solubilizing, washing, and manipulation of most preparations. This could have favored the detection of the 11S protein, too. No experiments were performed with calcium or magnesium salts of EDTA to purposely exclude the other known heat-labile complement requiring cations, namely C'1 and C'2 (6).

The absence of antigenicity of the 11S protein after heating suggests that it has aggregated or in some way changed its structure. Purified 11S preparations lost their capacity to precipitate with the anti-11S antiserum after heating at 56°C for as little as 3 minutes. Since all preparations of this protein are contaminated with the other immunoglobulins and to some extent soluble gamma globulin aggregates, further purification would seem necessary to ascertain this point. Its electrophoretic migration with the gamma globulins is also interesting but it does not appear to share antigenic determinates with any of the immunoglobulins.

The role of the 11S protein in the human complement system has not yet been thoroughly investigated. The agglutination of the intermediate EAC'11S by these antisera provides further evidence that the intermediate can exist as has already been demonstrated hemolytically by Müller-Eberhard with the R11S reagent (4). Preliminary studies with agglutination by these antisera of *in vivo* sensitized cells obtained from patients with autoimmune hemolytic anemias suggests that either this intermediate exists *in vivo* or that these antisera can still react with the 11S protein in complexes that form subsequent to the EAC'11S step. Work is also in process regarding this point by forming complement intermediates with human serum using the sensitized sheep cell system.

Lepow suggested that C'1 activity requires three components which can be separated by chromatography and he designated the first component C'1q (1). Since chromatography of an R11S in a Na<sub>2</sub> EDTA medium lacked C'1q, the identity of C'1q and the 11S protein was suggested. Whether Na<sub>2</sub> EDTA and low temperature (4°C) combine to constitute an abnormal milieu responsible for dissociation of a C'1 complex cannot be stated.<sup>5</sup> It has been shown by DeLooze *et al.* (11) that the rapid decay of the intermediate EAC'1 is reduced at 37°C. Further experiments are required to determine the true role of the at protein in this reaction.

<sup>5</sup> I. H. Lepow has presented preliminary evidence suggesting that the 11S protein is part of a macromolecular complex in native serum and that low temperature and Na<sub>2</sub> EDTA may serve to dissociate this complex. First Complement Workshop, *Science*, 1963, **141**, 738.

## SUMMARY

Rabbit anticryoprotein and anticomplement antisera recognized a heat-labile antigen in normal human serum. This antigen best fitted the previously described 11S protein because of its presence in fresh human serum, euglobulin, and purified 11S preparations and its absence in heated serum, R11S, and pseudoglobulin preparations. The 11S hemolytic activity correlated well with the presence of this heat-labile antigen in the 11S region in sucrose density gradient ultracentrifugation and in the gamma globulin region on zone electrophoresis. It could be identified as a single component in the gamma globulin region in immunoelectrophoresis. The intermediate complex EAC'11S was lysed by R11S reagents and agglutinated by these antisera. The antisera also agglutinated a human complement-binding Rh-positive cell system if the 11S protein had been previously bound.

The authors wish to thank Dr. Marion Waller for generously supplying the Ripley serum. The authors are indebted to Mrs. Phyllis Kasdon and Miss Gloria Dietz for technical assistance.

*Addendum.*—Since submission of the manuscript, the 11S protein has also been demonstrated in normal human serum submitted to phosphate immunoelectrophoresis. Furthermore, rabbit antisera prepared against “partially purified 11S protein” also recognize the same 11S antigen demonstrated by the anticryoprotein and anticomplement antisera utilized in this study.

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