

RHEUMATOID FACTOR PROPERTIES OF HYPERIMMUNE RABBIT SERA*

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Serum factors closely mimicking the human rheumatoid factor (RF) have been induced in rabbits by prolonged immunization with bacterial antigens (1, 2) and with soluble antigens (3, 4). A previous report concerned studies of a rheumatoid factor-like substance (RFLS) in sera of rabbits immunized with killed *Escherichia coli* or *Bacillus subtilis* (2). The RFLS was associated with the macroglobulin fraction of serum and possessed the properties of antibody specific for autologous gamma globulin but with cross-reactivity against human gamma globulin. Indirect evidence suggested that the RFLS reacted preferentially with immune complexes or with denatured gamma globulin and that the stimulus for its production might be the alteration of autologous gamma globulin which results from protracted immune complex formation *in vivo*.

The present report includes further studies of the RFLS induced by hyperimmunization with bacterial antigens. After continuous immunization for more than 1 year, the RFLS was consistently associated with the high molecular weight fraction of serum and was separable from the bulk of antibacterial antibodies by cellulose column chromatography or by sephadex G-200 filtration. With the use of rabbit isohemagglutinin-sensitized cells, there was evidence for some isospecificity of certain RFLS, and the part of rabbit gamma globulin reactive with the RFLS could be removed by pepsin digestion.

Materials and Methods

1. Immunization of animals with formalin-killed bacteria (*Escherichia coli*, *Bacillus subtilis*, or *Salmonella typhimurium*) was accomplished by intravenous injections 3 times weekly. (See reference 2 for details.) Bacteria were cultured on synthetic media. Sterile sera were stored at either 0–4°C or –20°C.

2. Serological methods for quantitating RFLS were: (a) hemagglutination of tanned sheep erythrocytes coated with heated (70°C, 10 minutes) rabbit gamma globulin (2), or (b) hemagglutination of rabbit erythrocytes which had been sensitized with incomplete rabbit isoag-

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glutinin sera. The isospecific antisera were anti-G (Kellner, 5) and anti-A (Cohen, 6).¹ Sensitization was accomplished by incubating a 1 per cent suspension of washed rabbit erythrocytes of the appropriate antigenic type with an equal volume of diluted isoagglutinin for 45 minutes at 37°C. After incubation, the cells were washed 3 times with cold isotonic saline and suspended as 0.25 per cent cells. After serial dilution of test sera in 0.5 ml volumes, 0.5 ml of the sensitized cells were added to each tube and incubated at 0-4°C overnight. End-points were read as macroscopic agglutination after gentle tapping of tubes.

The highest dilution of isoagglutinin sera which resulted in 4+ antiglobulin reaction (duck anti-rabbit serum antiserum) was used for sensitizing cells.

3. Pepsin digestion of rabbit gamma globulin (water soluble euglobulin purified by ammonium sulfate precipitation; reference (7) was according to methods of Nisonoff *et al.* (8, 9). The pepsin digests were precipitated first at 12.5 gm per cent sodium sulfate, and the precipitate was discarded. The precipitate resulting from 18.8 per cent sodium sulfate was dissolved in distilled water and dialyzed against pH 8.0 buffered saline. Crystalline pepsin was obtained from Nutritional Biochemicals Corp., Cleveland.

4. Nitrogen estimations were done by the Folin-Ciocalteu reaction using rabbit gamma globulin standards (10).

5. *Preparative techniques:* Zone centrifugation utilized gradients prepared by permitting overnight diffusion of successive 1 ml layers of 40, 30, 20, and 10 per cent sucrose. After layering of test samples on top of the gradient and centrifugation at 35,000 RPM (39 SL rotor) in a Spinco model L ultracentrifuge for 12 to 18 hours, successive (0.5 ml) fractions were obtained of the effluent from a pin hole in the bottom of the cellophane tubes.

Diethylaminoethyl (DEAE) cellulose chromatography utilized a stepwise gradient from pH 8.0, 0.02 M to pH 4.6, 0.15 M phosphate buffers. Columns of DEAE (Carl Schleicher & Schüll Company, Keene, New Hampshire) were prepared according to the instructions of Fahey (11).

Sephadex G-200 filtration was performed on columns 12 inches long with an internal diameter of $\frac{3}{4}$ inches, equilibrated and eluted with pH 7.0, 0.05 M phosphate buffer.

6. Analytical sedimentation studies were performed in a Spinco model E ultracentrifuge. Sedimentation values were not corrected for concentration effects.

7. *Gel diffusion studies:* Ouchterlony-type double diffusion studies were performed in Petri dishes containing 25 ml of 0.70 per cent agar (pH 7.6 veronal, 0.33 M glycine). Immunoelectrophoretic analysis was according to the method of Grabar and Williams (12). Lantern slides (82 × 102 mm) were coated with 1 per cent agar in pH 8.6 barbital buffer (0.05 ionic strength). A current of 25 ma per plate was applied for 4 to 6 hours.

The antiserum used for gel diffusion analyses was duck antiserum against whole rabbit serum.

RESULTS

Fig. 1 illustrates the development of bacterial agglutinins and the RFLS in a single rabbit immunized with *B. subtilis*. The relatively delayed appearance of the RFLS has been consistently observed. With cessation of immunization after 33 weeks, a gradual decrease in both serological properties followed. Eighteen weeks later, reinjection of the bacteria resulted in a prompt several-fold increase in the bacterial and tanned cell agglutination titers. The serum

¹ The author is indebted to Dr. Aaron Kellner for supplying the typing reagents necessary for grouping rabbits for the G system and to Dr. Carl Cohen for providing the 2 anti-A sera used in these studies. The production of anti-G antisera was according to Kellner's method.

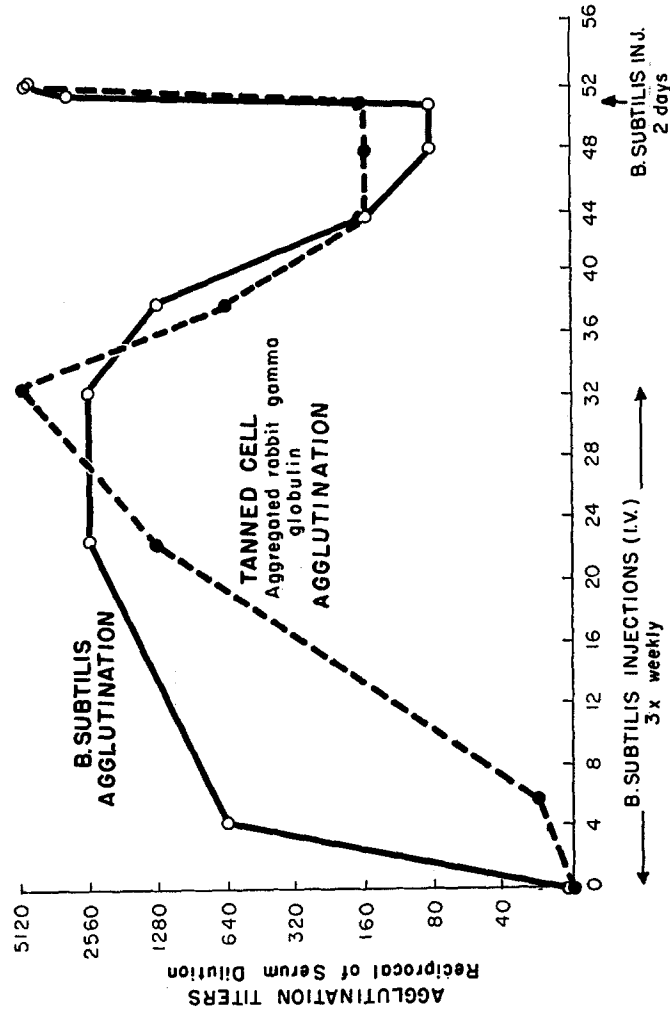


FIG. 1. Serological responses of a rabbit (B4) immunized with *B. subtilis*, demonstrating the effects of cessation of immunization and subsequent secondary antigen administration. (Time intervals in weeks.)

after this secondary stimulus was subjected to zone centrifugation (see Fig. 2). The RFLS was present in the bottom of the tube separate from the bulk of the antibacterial antibodies. Fig. 3 illustrates a comparable study of serum from a rabbit immunized with *E. coli* for a period in excess of a year. The relative

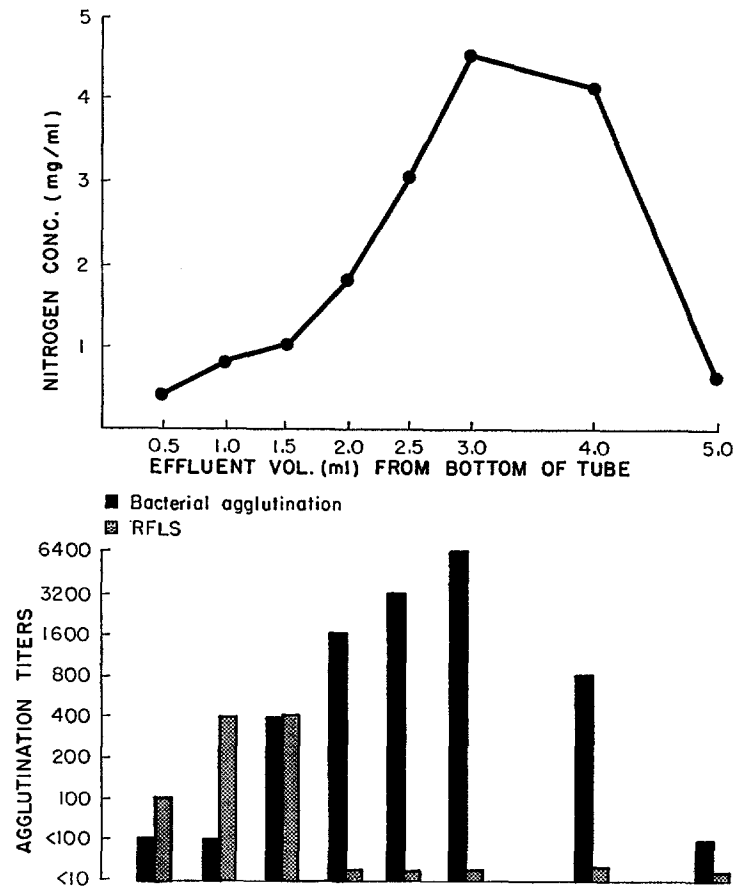


FIG. 2. Zone centrifugation of the serum of a rabbit (B4) which received a secondary stimulus (injection of *B. subtilis*).

separation of the RFLS and *E. coli* agglutinins is evident. Gel filtration (sephadex G-200) of a serum from a rabbit immunized with *E. coli* and *S. typhimurium* resulted in similar separation of the 2 serological properties (Fig. 4).

Fractionation of the serum of an *E. coli*-immunized rabbit with DEAE cellulose is depicted in Fig. 5. The bacterial agglutinins were detected only in the fall through fraction, while the RFLS was obtained in the fraction eluted with pH 4.6, 0.15 M phosphate.

The above fractionating procedures were used in preparation of partially purified RFLS for further studies. Fig. 6 illustrates an immunoelectrophoretic analysis of B4 serum and the heaviest fraction of B4 serum obtained from zone centrifugation. This fraction, labeled dens. grad. I (density gradient I),

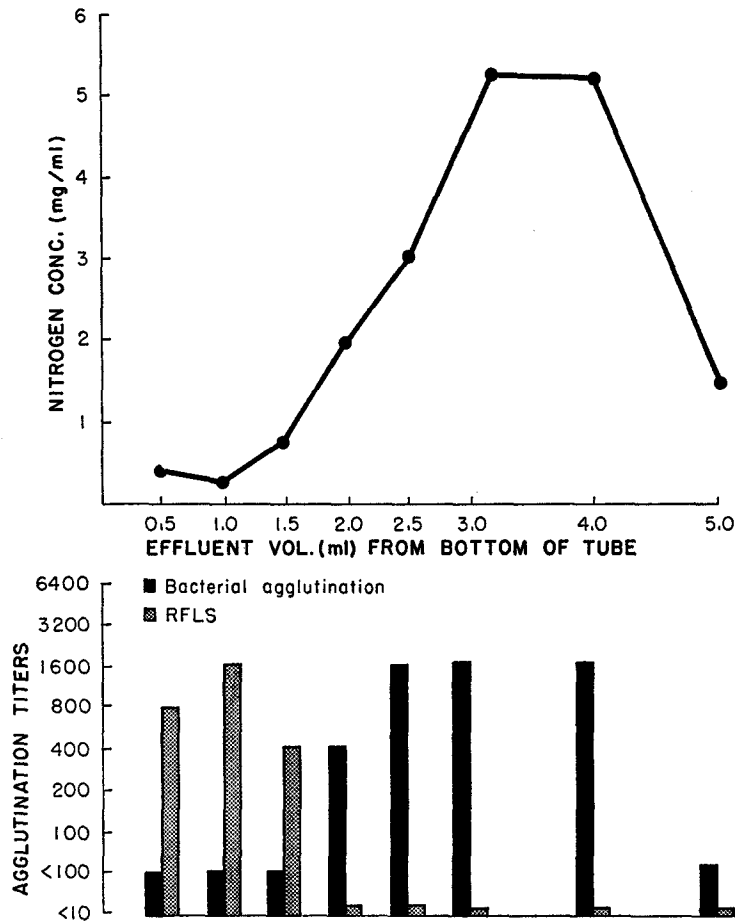


FIG. 3. Zone centrifugation of the serum of a rabbit (E35) which was immunized (3 times weekly, intravenously with *E. coli* for 14 months.

was free of detectable antibacterial antibodies. Only two precipitin lines with duck anti-whole rabbit serum antiserum were visible, one each in the α and β regions. A preparation which resulted from euglobulin precipitation and fractionation on DEAE cellulose (fraction V) was studied by immunoelectrophoretic analysis and is illustrated in Fig. 7. Two precipitin arcs in the γ - β regions are noted. Fig. 8 is a sedimentation pattern of this same preparation

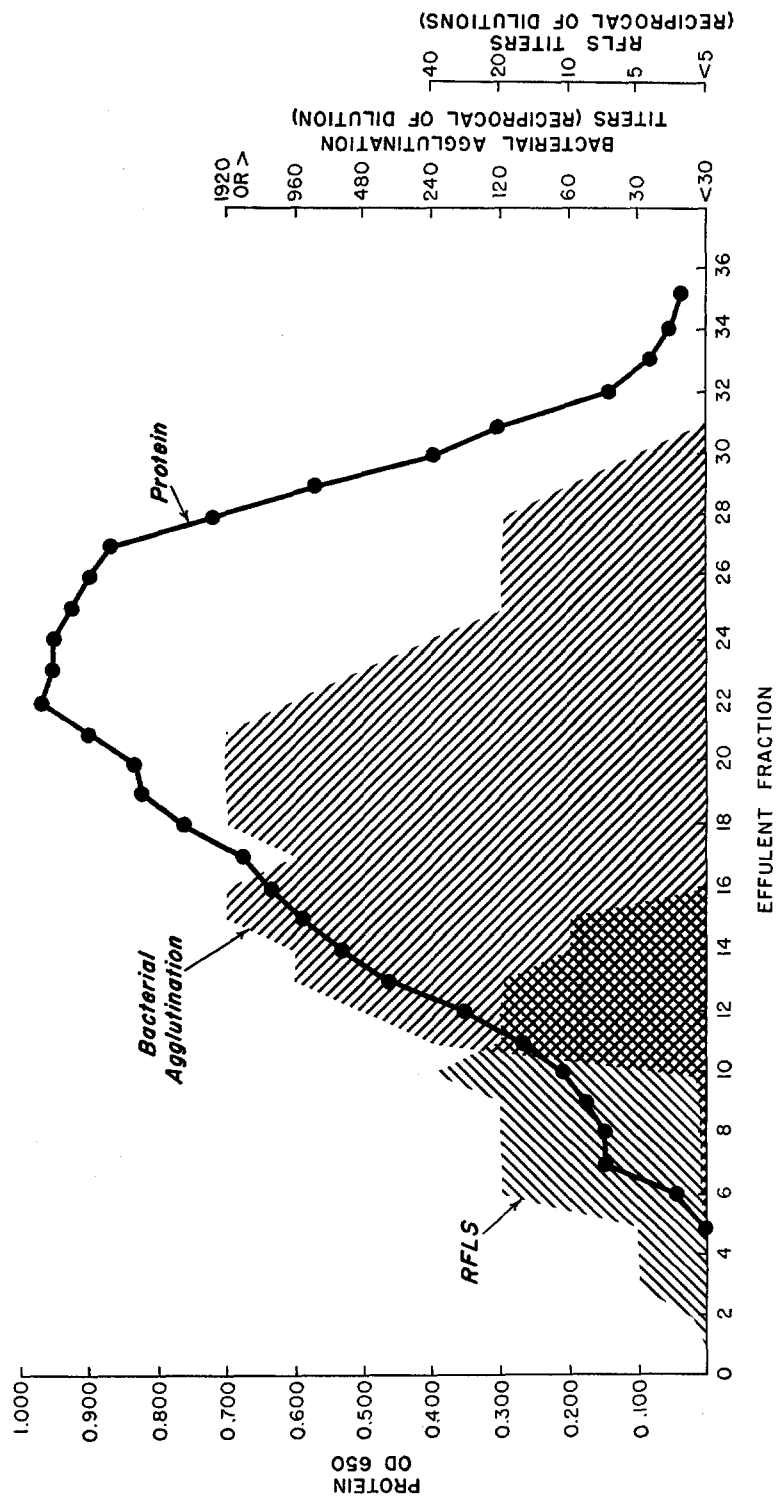


FIG. 4. Gel filtration (sephadex G-200) of the serum of a rabbit (1-42) immunized with *E. coli* and *S. typhimurium*. 5 ml of serum was applied to the column and 0.5 ml effluent fractions were collected.

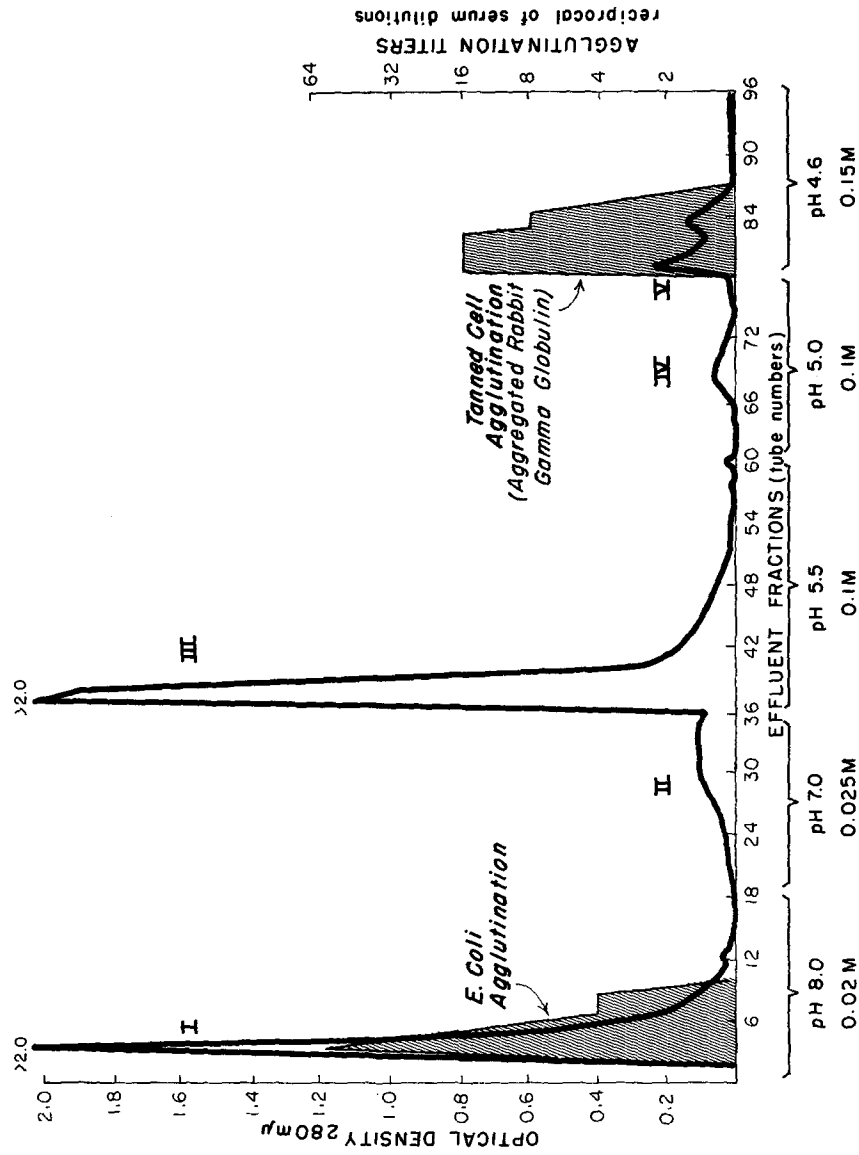


FIG. 5. Fractionation of an RFLS serum (E19) on DEAE cellulose.

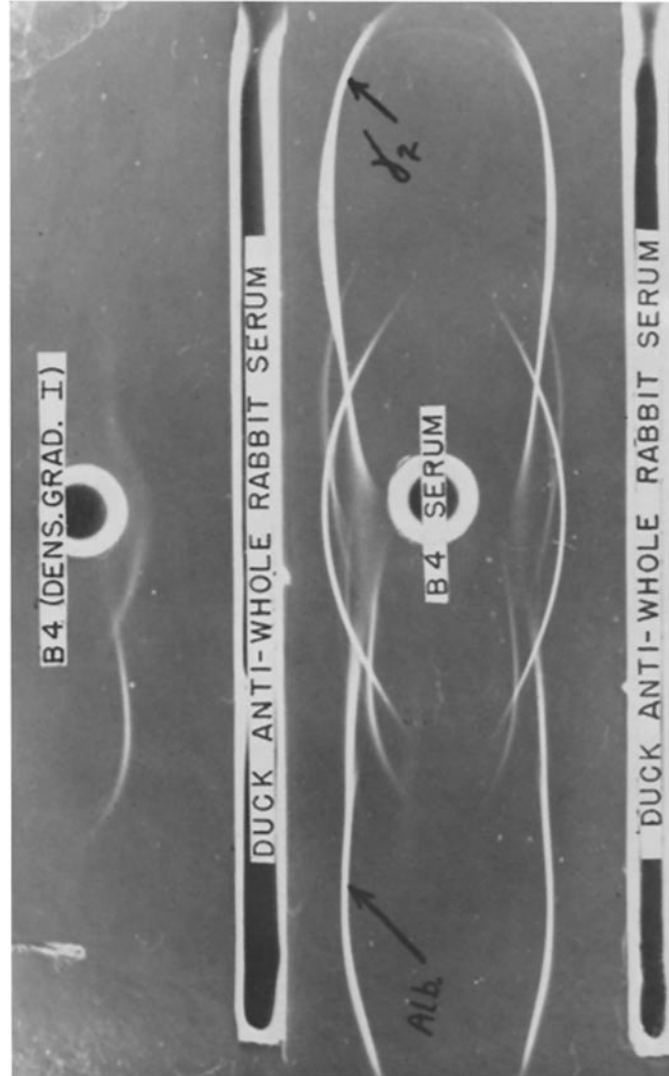


FIG. 6. Immunoelectrophoretic analysis of an RFLS serum and the partially purified RFLS prepared by zone centrifugation.

(DEAE fraction V of serum B4 euglobulin). Two main components are visible. The faster major peak had an S_{20} value of 17.5, and the slower component an

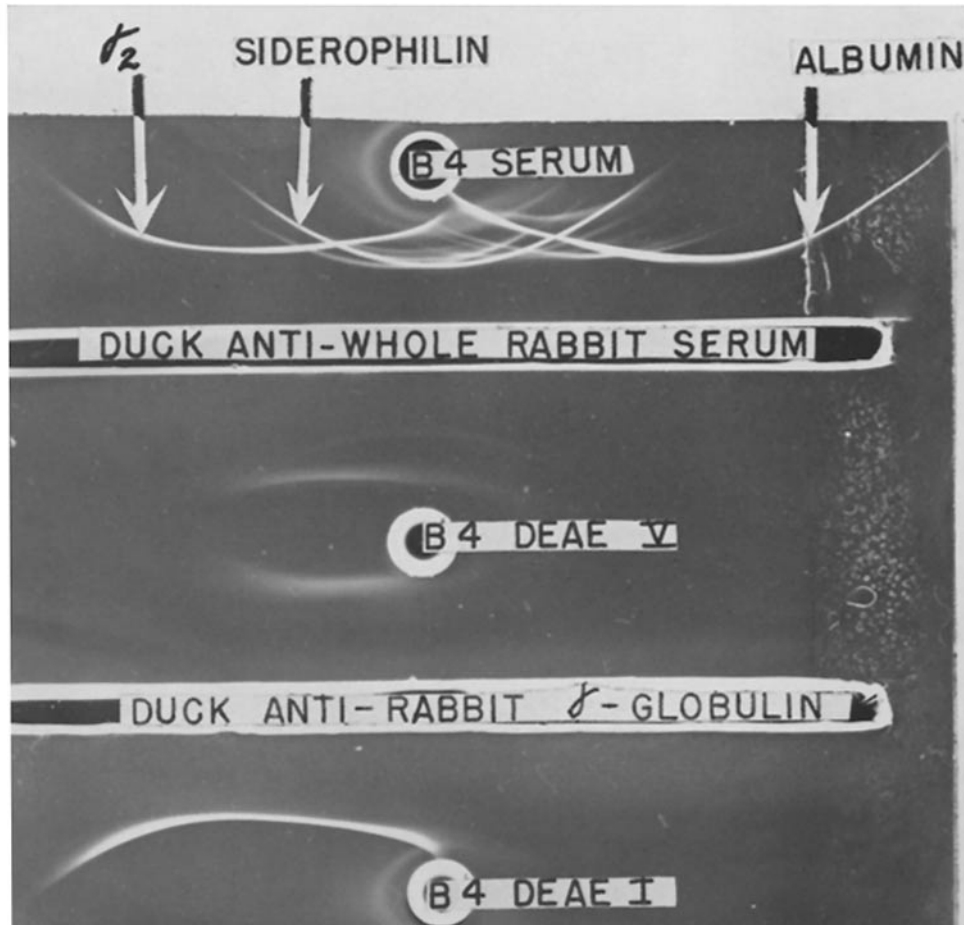


FIG. 7. Immunoelectrophoretic analysis of a rabbit serum with RFLS properties and fractions of the serum obtained by DEAE cellulose chromatography.

B4 DEAE I, fall through fraction; B4 DEAE V, fraction of euglobulin preparation which eluted from DEAE column at pH 4.5, 0.15 M phosphate.

S_{20} value of 6.4. (The agglutination titer of this preparation for isoagglutinin-sensitized cells was in excess of the titer of whole serum.)

In view of the previously noted similarities of the RFLS to the human RF and the varied serological specificity of the latter for different genetic types of human γ -globulin, it was of interest to investigate the rabbit RFLS for iso-

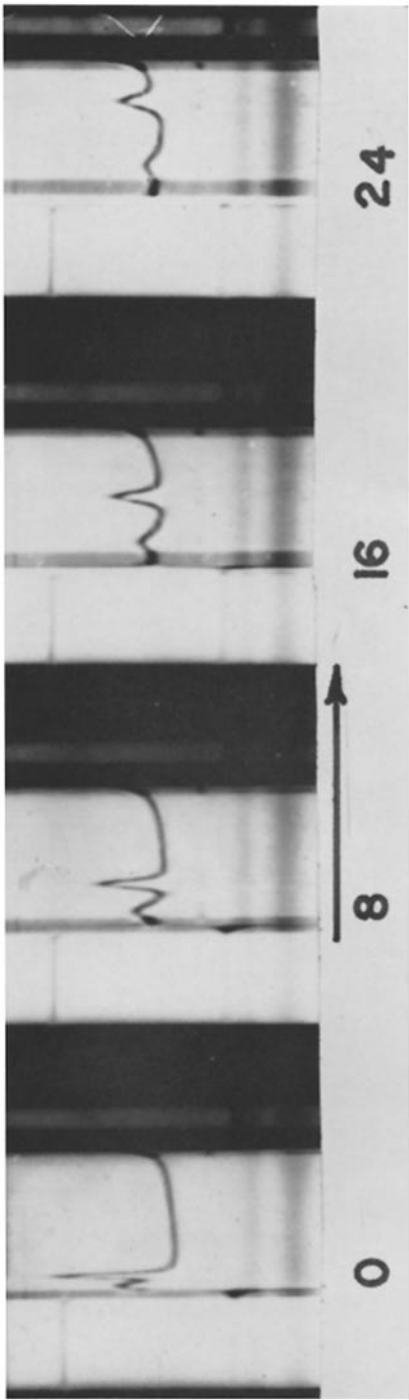


Fig. 8. Sedimentation pattern of a partially purified preparation of the RFLS (Serum B4 euglobulin—DEAE fraction V). Exposure intervals (minutes) as indicated at 47,660 RPM. Arrow indicates the direction of sedimentation. The more rapidly sedimenting component had an S value of 17.5.

specificity. Table I summarizes the results of testing 9 RFLS sera with 4 different isohemagglutinin coats. With some RFLS sera, equivalent titers were noted with all 4 coats but certain sera, such as E₁₅, were non-reactive with 1 or more coats. The possibility that this pattern of reactivity was determined by known allotypic antigens of rabbit γ -globulin was not supported by allotypic studies carried out by Dr. Sheldon Dray. Isoagglutinin coats G13 and C239 had identical allotypes but divergent reactivity with 4 of the RFLS sera.

The data in Table II demonstrate that the pepsin-digested gamma globulin of an isohemagglutinin serum is approximately equal to the undigested globulin

TABLE I
Reaction of a Panel of RFLS Sera with 4 Different Rabbit Iso-Hemagglutinin Coats

	Rabbit isoagglutinin sera			
	G ₁₃	K ₁	C ₂₃₉	C ₁₀₇
Normal rabbit serum (E ₁₂).....	0	0	0	0
Test rabbit sera				
E ₈	1280	1280	1280	1280
E ₁₅	320	640	0	320
E ₁₉	320	640	40	320
No. 2.....	640	640	640	640
E ₁₀	40	80	0	20
No. 9.....	320	640	320	160
B ₄	160	320	160	320
E ₁₈	80	80	0	0
E ₂	160	160	0	80

Figures are reciprocals of agglutination titers.

in coating erythrocytes for antiglobulin agglutination. When equal amounts of the pepsin-treated and undigested globulins were used to sensitize cells, there was a marked difference in the agglutination by RFLS sera (see Table III). Fig. 9 demonstrates that the pepsin-treated globulin lacks antigenic determinates present in the whole gamma globulin. Data in Table IV compare the absorbing capacities of immune precipitates which were derived from the whole gamma globulin of a rabbit antiovalbumin serum and from the same globulin which had been treated with pepsin. (Precipitates were formed at equivalence.) Absorption of the diluted RFLS serum with 0.12 mg N precipitate from undigested globulin caused a threefold reduction in titer. Precipitate from pepsin-digested globulin did not significantly remove the RFLS when 5 times the above quantity was used.

DISCUSSION

Previous studies and the data reported herein demonstrated that the rabbit RFLS (induced by bacterial hyperimmunization) has consistently and exclusively been associated with the macroglobulin fraction of serum. When the

TABLE II
Comparison of Sensitizing Capacities of Pepsin-Treated and Untreated Rabbit γ -Globulin (Derived from a Rabbit Isohemagglutinin Serum) for Antiglobulin Agglutination

Treatment of isoagglutinin globulin	Concentration isoagglutinin globulin <i>mg N/ml</i>	Reaction with antiglobulin serum
None	0.28	4+
	0.14	4+
	0.07	3+
	0.035	2+
	0.018	2+
	0.009	1+
Pepsin digestion	0.28	4+
	0.14	4+
	0.07	3+
	0.035	2+
	0.018	1+
	0.009	1+

Equal volumes of the globulin and 1 per cent rabbit erythrocytes were mixed and incubated (see Materials and Methods).

TABLE III
Comparison of the Sensitizing Capacities of Pepsin-Treated and Untreated Rabbit γ -Globulin (Derived from a Rabbit Isohemagglutinin Serum) for Agglutination by 6 RFLS Sera

Reagent for "Coat"	Titers with panel of RFLS sera					
	E47	E27	E43	E37	E31	E36
G13 serum 1:100 dil.	320	160	80	160	160	320
G13 γ -globulin 0.14 mg N/ml	640	320	160	320	160	640
G13 pepsin-digested γ -globulin 0.14 mg N/ml	<10	40	<10	40	<10	<10

duration of immunization exceeded 1 year or when a secondary response resulted in rapid and several-fold increase in the RFLS, the anti-gamma globulin activity was present only in the high molecular weight fractions of the sera studied (see Figs. 2-4). (Unsuccessful attempts to demonstrate 7S

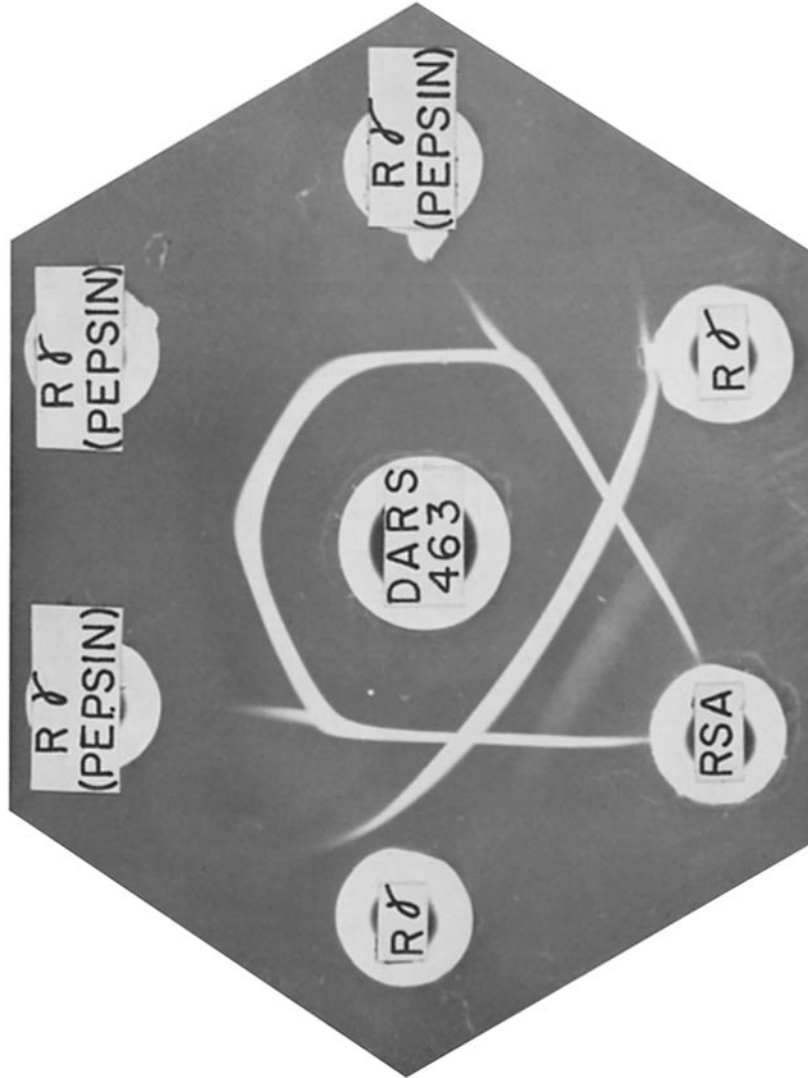


FIG. 9. Gel diffusion pattern of pepsin-digested and untreated rabbit γ -globulin derived from a rabbit isoagglutinin serum (GI3). RSA, rabbit serum albumin. DARS, duck anti-whole rabbit serum antiserum.

factors with anti- γ -globulin specificity included the use of rabbit erythrocytes sensitized with pepsin-treated rabbit isoagglutinins,—a system comparable to that utilized for the demonstration of 7S human RF; reference 13.) This is somewhat at variance with studies which demonstrate, in general, that prolonged immunization and secondary antigenic stimulation result preferentially in production of low molecular weight antibody (7S γ -globulin) (14–19). This generalization was applicable to the antibacterial antibodies induced in the present studies. In none of the hyperimmune sera studied was there more than trace amounts of antibacterial antibodies in the high density fraction. The

TABLE IV
Absorption of RFLS Serum with Ovalbumin-Antiovalbumin Precipitates Derived from Gamma Globulin and Pepsin-Treated Gamma Globulin Fractions of Rabbit Antiserum

	Precipitate N used for absorption	Agglutination titer for rabbit isoagglutinin sensitized cells
Unabsorbed serum (142)	—	640
Absorbed with immune precipitate formed from whole gamma globulin	0.12	80
	0.30	20
	0.60	20
Absorbed with immune precipitate formed from pepsin-treated gamma globulin	0.12	640
	0.30	320
	0.60	640

The washed precipitates were incubated with 1 ml of a 1:10 dilution of 142 serum at 0–4°C overnight.

basis for the persistence of the RFLS as high molecular weight γ -globulin (or the basis for exclusive association of human anti-typhoid O agglutinins with the 19S fraction) is not known. The nature of the antigen (rabbit γ -globulin) cannot alone explain this phenomenon since anti- γ -globulin antibodies induced in rabbits by immunization with denatured or enzyme-treated autologous γ -globulin or by immunization with horse spleen ferritin in adjuvant have been in large part 7S antibodies (4, 20). Rather it seems more likely that the persistence of RFLS as 19S antibody is related to one or more of the following: (a) the nature of the denaturation of rabbit γ -globulin which results from immune complex formation, (b) the carrier properties of the particulate antigens (bacteria) used in the present studies, or (c) the intravenous route of immunization. (Most experiments utilizing injected autologous γ -globulin as the stimulus for production of anti- γ -globulin factors have incorporated the γ -globulin or its digestion products into complete Freund's adjuvant for subcutaneous or intramuscular injection; references 20–22.)

Attempts to obtain the RFLS in high yield free of other serum proteins have been difficult but there seems little doubt, from the studies herein reported, that the RFLS is present in the fraction of serum which has an uncorrected S value of 17.5. With immunoelectrophoretic analysis, this serum component had properties somewhat different from human γ_{1M} -globulin (less diffuse and somewhat more anodal in its distribution). Similar immunoelectrophoretic patterns of purified RFLS were obtained when guinea pig anti-rabbit γ -globulin and sheep anti-whole rabbit antisera² were used instead of the duck antiserum. The macroglobulin component was not clearly demonstrable in immunoelectrophoretic analyses of whole rabbit sera.

Some isospecificity of selected RLFS sera was apparent from data in Table I. The failure to relate the isospecificity of RFLS sera to the known allotypes of rabbit γ -globulin and the apparent specificity of the RFLS for the part of rabbit γ -globulin which is removed by pepsin digestion are consistent observations. Studies to date suggest that pepsin digestion of rabbit γ -globulin removes Porter fraction III (8, 23), and the known allotypes have been associated with Porter fractions I and II (24-26). The human RF has been shown to react with Porter fraction III of rabbit γ -globulin (27, 28) and with the papain-split fragment of human γ -globulin which corresponds to Porter fraction III (29). Recent studies, however, have demonstrated reactivity of 7S human anti- γ -globulin factors with pepsin-treated human γ -globulin (13). Although the main reactivity of the RFLS seemed directed at Porter fraction III, the observation that 2 sera (E27 and E37 in Table III) displayed residual low tiers of agglutinins for cells coated with pepsin-digested γ -globulin could be cited as evidence for a small population of antibodies in these sera with specificity directed against Porter fractions I, and II. With certain reagents, hemagglutination inhibition studies permitted separation of normal rabbit sera into those that inhibit in high dilution and others which are not inhibitory in low dilution (30). It is possible that such studies will permit the classification of rabbit γ -globulin into genetic types on the basis of antigenic variations in Porter fraction III. This system of study would be analogous to the Gm typing of human γ -globulin where hemagglutination inhibition is the basis for differentiation, and the antigenic determinates responsible for inhibition of agglutination are present on the human equivalent of Porter fraction III (31, 32).

SUMMARY

The rheumatoid factor-like substance (RFLS) induced by hyperimmunization of rabbits with bacteria was consistently and exclusively associated with the macroglobulin fraction of the sera studied. The RFLS is separable from the bulk of serum proteins by zone centrifugation, DEAE cellulose chromatography, and gel filtration. Partially purified preparations of the RFLS were

² The sheep antiserum was kindly supplied by Dr. J. Thorbecke.

studied by immunoelectrophoretic analysis and analytical ultracentrifugation. Certain RFLS sera demonstrated selective reactivity with one or more rabbit isohemagglutinin antibodies. Pepsin treatment of rabbit γ -globulin removed or greatly diminishes its capacity to react with the RFLS.

These experiments were carried out with the competent technical assistance of Phyllis Kasdon and Mabel Wong.

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