CHARACTERIZATION OF THE "REACTANT" (GAMMA GLOBULIN FACTOR) IN THE F II PRECIPITIN REACTION AND THE F II TANNED SHEEP CELL AGGLUTINATION TEST

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A comprehensive review of the serological reactions in rheumatoid arthritis has been published recently (1). In general, these tests give positive results in the majority of patients with rheumatoid arthritis while only a small percentage (1 to 3 per cent) of controls react positively. The most extensively studied system is the sensitized sheep cell test (2, 3). In this procedure sheep erythrocytes exposed to a subagglutinating concentration of rabbit anti sheep cell hemolysin are agglutinated by most rheumatoid sera and are termed sensitized cells.

Heller and his associates, in their studies of the sensitized sheep cell test, demonstrated that Cohn fraction II (gamma globulin) of human sera inhibited the agglutinating activity of a serologically positive rheumatoid serum (4). As development of this observation, further tests have been described (F II tanned sheep cell agglutination test (4), F II precipitin test (5), and the F II latex fixation test (6)) and considerable progress has been made towards an understanding of the basic mechanism involved in all the serological tests for rheumatoid arthritis.

In brief, this basic mechanism appears to be a reaction between a material present in most rheumatoid sera which is termed the "rheumatoid factor" and some component of gamma globulin—the "reactant." In the sensitized sheep cell test, rabbit gamma globulin (anti sheep cell hemolysin or the amboceptor) constitutes the "reactant." In more recently studied systems (the F II tanned sheep cell agglutination test, the F II precipitin test, and the F II latex fixation test), the "reactant" is contained in a preparation of human gamma globulin (F II).

Franklin *et al.* demonstrated that the "rheumatoid factor" is a gamma globulin with a sedimentation constant of 19, which probably is contained in a complex (7, 8). The intact complex has a sedimentation constant of 22 and under certain conditions dissociates into two gamma globulin components with sedimentation constants of 19 and 7.

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Relatively little is known of the "reactant." The most promising system for study of this factor has been the F II precipitin reaction. Not all preparations of human gamma globulin precipitate when added to rheumatoid serum. However, a previously non-precipitating preparation can be made reactive by a variety of treatments; *i.e.*, exposure to an alkaline pH, heating to 63° for 10 minutes (7, 9, 10), and treatment with dilute formalin (11). In a precipitin study with even a highly reactive preparation of gamma globulin, several milligrams of the material will, when added to a rheumatoid serum, yield only micrograms of precipitate. Thus, the "reactant" would appear to constitute a very small portion of the whole gamma globulin. Franklin *et al.* noted that a precipitating component of gamma globulin preparations sedimented rapidly with short periods of ultracentrifugation, indicating that the active material was aggegated gamma globulin (7).

In the present study, sodium sulfate fractionation has been found to be a reliable method of concentrating the "reactant" from whole gamma globulin–Cohn fraction II in most cases. Further evidence will be offered which suggests that the "reactant," at least as it applies to the F II precipitin and F II tanned sheep cell agglutination reactions, consists of aggregates of gamma globulin molecules.

Materials and Methods

1. The sources of gamma globulin were commercial pooled human F II (Squibb) and gamma globulin isolated from the sera of healthy human donors by electrophoresis convection (12). The electrophoretic character of the material in each solution was determined by both moving boundary and paper electrophoretic techniques. Unless otherwise stated, preparations of gamma globulin were not purposely altered by heat or other treatments.

2. Fractionation of gamma globulin with sodium sulfate was accomplished by successive additions of 20 cc. portions of 2.18 molar sodium sulfate to 100 cc. of 2 per cent gamma globulin dissolved in buffered saline (pH 8.0). Six different fractions (fractions SS_1 to SS_6) were precipitated at molarities of sodium sulfate listed in Fig. 2. The precipitates were dissolved in small volumes (2 to 10 cc.) of buffered saline (pH 8.0), dialyzed against the same buffer for 12 to 24 hours, and then centrifuged for 2 to 3 hours at 3000 R.P.M.

3. Sera from patients with rheumatoid arthritis were obtained from patients in the Edward Daniels Faulkner Arthritis Clinic and the Presbyterian Hospital. Control sera were collected from healthy donors.

4. Buffered saline (pH 8.0) (4) was used as the diluent of all material unless otherwise noted.

5. F II tanned sheep cell agglutination tests were performed according to the method of Heller (4).

6. "F II tanned cell inhibition" was measured by the degree to which a given preparation of gamma globulin could inhibit the F II tanned sheep cell agglutinating activity of a known positive rheumatoid serum. This procedure was performed by serially diluting the test material in 0.4 cc. of buffered saline (pH 8.0). A diluted rheumatoid serum (0.1 cc.) with an F II tanned sheep cell agglutinating titer of 1:64 was added to each tube, making the titer of the resultant 0.5 cc. in each tube 1:12. After incubation at 37°C. for 30 minutes, a 0.25 per cent suspension of F II coated sheep cells was added, as in the routine Heller test, and the endpoint read as complete inhibition of agglutination.

7. F II precipitin reaction was performed essentially as described by Epstein *et al.* (5). Aliquots of test serum (0.25 cc. unless otherwise stated) were added to calibrated 8 cc. centri-

fuge tubes. Varying amounts of gamma globulin or fraction thereof were placed in the tubes and buffered saline (pH 8.0) added so that the reaction volume was 5.0 cc. The tubes were incubated at 37°C. for 30 minutes, then at 4°C. for 48 hours, after which the precipitates were packed by centrifufation at 2000 R.P.M. in a refrigerated centrifuge. The precipitates were resuspended and washed three times with 7.5 cc. of buffered saline. After the final washing and centrifugation, the precipitates were dissolved in a few drops of 1 molar NaOH and analyzed for nitrogen.

8. Nitrogen content was determined by the Folin method (13), gamma globulin standards (nitrogen determined by Kjeldahl) being included with each series of determinations.

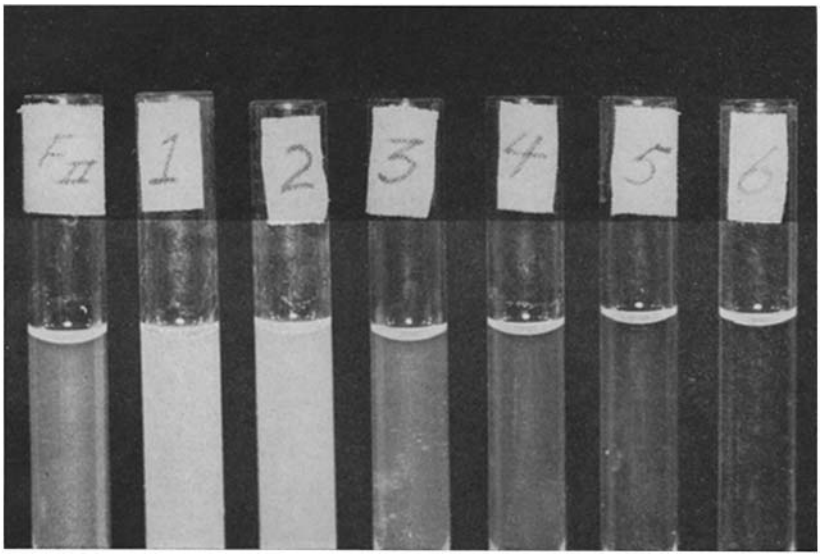

FIG. 1. Photograph of whole F II and sodium sulfate fractions of F II, demonstrating the opalescence of sodium sulfate fractions SS_1 and SS_2.

9. Hexose was determined by the anthrone reaction (14) using glucose standards.

10. Sedimentation studies were performed in a Spinco analytical ultracentrifuge (model E). S_{20} values were calculated at multiple protein concentrations and corrected to infinite dilution.

RESULTS

1. Sodium Sulfate Fractionation.—Fractionation of whole F II with sodium sulfate yielded six different fractions in most cases (see Materials and Methods). The slight to moderate opalescence of F II solutions was concentrated in the first two fractions (Fig. 1).[1] The yields of fractions SS_1 and SS_2 could be greatly increased by heating the F II to 63°C. for 10 minutes prior to fractionation.

[1] Tubes 1 and 2 contained all fractions SS_1 and SS_2, respectively, from 200 cc. of 2 per cent F II. Since the volume in each tube was 3 cc., this represented an approximate 60-fold concentration of these two fractions.

Some of the physicochemical properties of these fractions are summarized in Fig. 2. Studies of fraction SS_1 in the ultracentrifuge demonstrated, in addition to the usual 7S component, an asymmetrical peak with an s_{20} value of

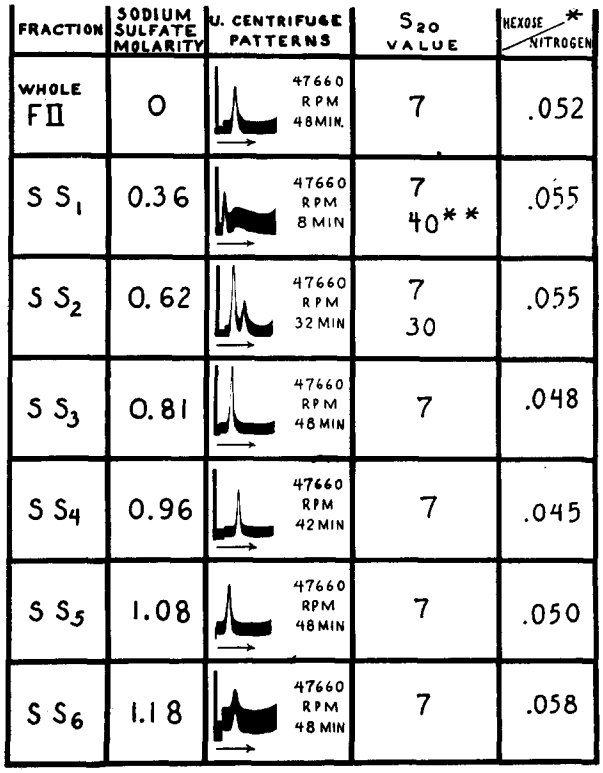

FIG. 2. Ultracentrifuge characterization of whole F II and sodium sulfate fractions (SS_1 to SS_6) of F II.

* $\frac{\text{Hexose (anthrone reaction)}}{\text{Nitrogen (Folin)}}$ ratio.

** See text.

approximately 40.[2] Studies of fraction SS_2 showed a fairly symmetrical component with a sedimentation constant of 30.[3] Fractions SS_3 through SS_6 did

[2] More precise characterization of the sedimentation velocity of fraction SS_1 was not possible since attempts at purifying the heavy component by refractionation or centrifugation resulted in irreversible precipitation.

[3] The heavy material in the original fraction SS_2 sedimented only twice as fast as the slower

not have detectable material heavier than 7S. Ultracentrifuge patterns of whole F II and fractions SS_1 and SS_2 at different intervals of time are shown in Fig. 3. In fraction SS_1 and to a much lesser extent in fraction SS_2, there was a heavy inhomogeneous material which sedimented rapidly during the early phases of centrifugation and did not resolve into distinct components. The solubility of this material was greatly reduced, since dialysis against physiological saline in the cold or attempts at concentration by centrifugation resulted in irreversible precipitation of much of it. As noted by Franklin *et al.*, the inhomogeneous gamma globulin, which can be separated from more slowly sedimenting components by short periods of ultracentrifugation, induced pre-

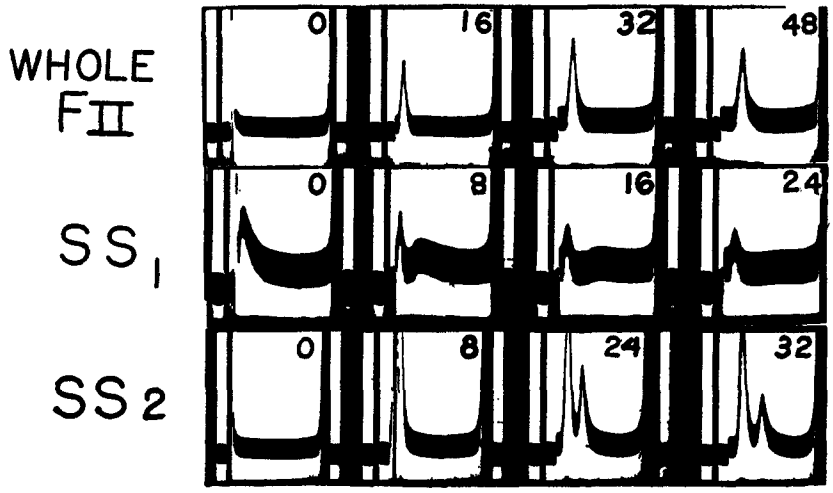

FIG. 3. Ultracentrifuge patterns of whole F II and fractions SS_1 and SS_2. The time interval (minutes) of each exposure is indicated. The centrifuge speed was 47660 R.P.M.

cipitate formation when added to a serologically positive rheumatoid serum (7). The striking opalescence of fractions SS_1 and SS_2 was due only in part to this very heavy material, for these fractions remained opalescent after the inhomogeneous substance had been removed by ultracentrifugation. The hexose to nitrogen ratios of the fractions were not significantly different, and are included since Müller-Eberhard *et al.* have demonstrated that the carbohydrate content of normal 19S gamma globulin is several times that of 7S gamma globulin (15). When fractions SS_3 through SS_6 were heated to 63°C.,

7S component. However, when this component was purified by differential centrifugation or salt fractionation, and the sedimentation constant redetermined, a value of 30 was obtained. Whether the value of 30 for the heavy component alone represented the actual sedimentation constant or whether further aggregation occurred in the process of refractionation is not known.

they became opalescent and sedimentation studies of these heated fractions demonstrated components heavier than 7S. The quantity of heavy material formed would thus seem to be dependent on the extent of aggregation of any 7S gamma globulin, rather than the select aggregation of a specific moiety present in whole gamma globulin.

Fractionation of gamma globulin (prepared by electroconvection) which did not react in the precipitin test did not yield fractions with demonstrable material heavier than 7S. Heating such preparations of gamma globulin to 63°C. for 10 minutes prior to fractionation induced opalescence and the first two sodium sulfate fractions of the heated material resembled the same fractions of whole F II gamma globulin. Salt fractionation appeared to be only a method of concentrating aggregated material as a function of its lowered solubility—not a means of producing molecular aggregation.

2. Precipitin Phenomenon.—The precipitating properties of whole F II and the sodium sulfate fractions of F II are illustrated in Fig. 4. Fractions SS_1 and SS_2 yielded large amounts of precipitate when added to rheumatoid serum while the remaining sodium sulfate fractions (SS_3 to SS_6) did not induce significantly more precipitate than was formed spontaneously in the serum. Heating the solution of F II prior to fractionation increased the reactivity of the whole F II, and fractions SS_1 and SS_2. The quantity of fraction SS_1 from unheated F II was insufficient for testing in this particular study.

A more detailed precipitin study is summarized in Fig. 5. The precipitin character of whole F II was intermediate between fractions SS_1 and SS_2 which showed marked precipitation and fractions SS_3 to SS_6 which had insignificant precipitating properties. A comparison of the precipitating properties of whole F II and fraction SS_2 in which larger quantities of the materials were added to rheumatoid serum is shown in Fig. 6. The precipitin curve with whole F II rose to a plateau and then in many cases declined with the addition of larger amounts of F II (5). In other experiments, the precipitin curves of fractions SS_1 and SS_2 ascended with diminishing steepness but did not reach plateaus in the range of concentrations studied.

With the use of a partition analytical ultracentrifuge cell, the heavy components in fractions SS_1 and SS_2 were separated from the still heavier inhomogeneous aggregated material. These separated components, *i.e.* components with s_{20} values of approximately 30 and 40, induced precipitate formation readily when added to a serologically positive rheumatoid serum.

Vaughan had noted, in the F II precipitin reaction, that the precipitate yield was greater with large reaction volumes; *i.e.*, with greater dilution of the reacting materials (16). This phenomenon is illustrated in the upper part of Fig. 7, which compares the precipitin curves with whole F II at two different reaction volumes. In the bottom part of the figure is a similar study with fraction SS_2, which demonstrates the absence of the volume effect. A more

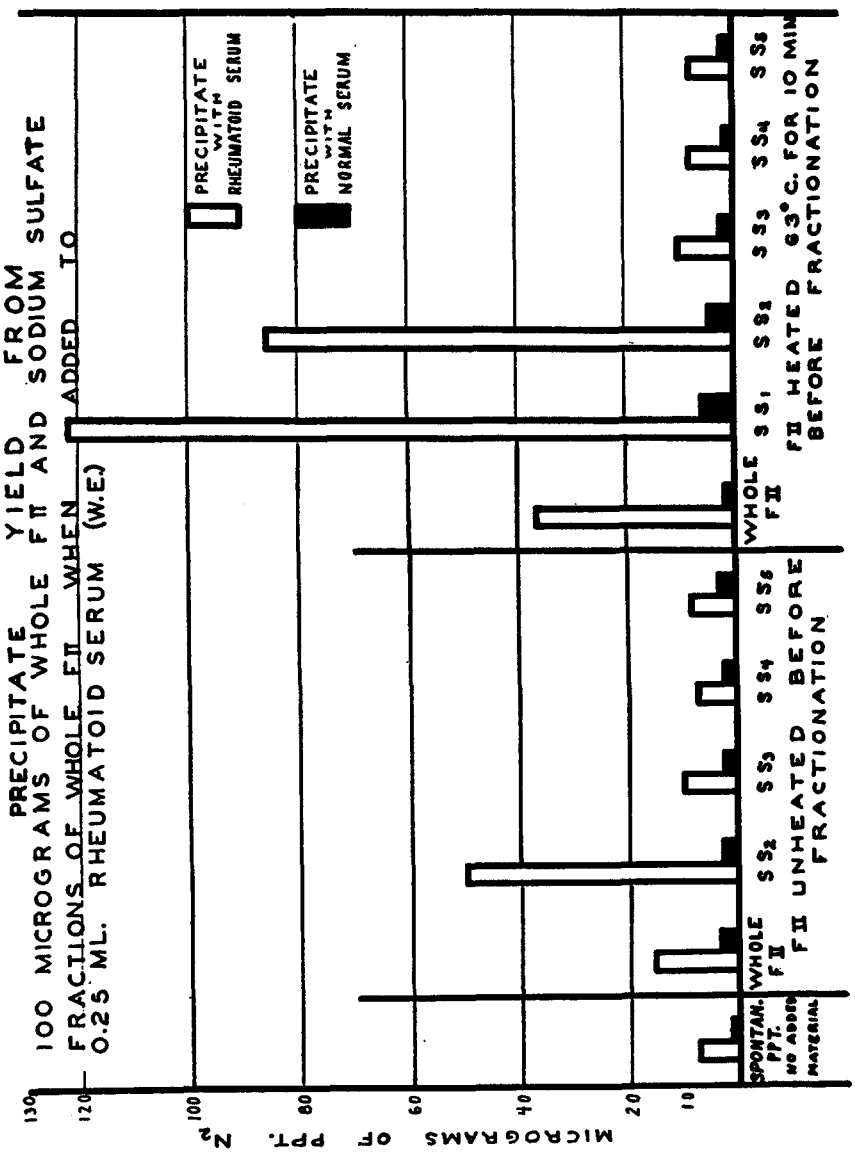

FIG. 4. Precipitin study with whole F II and sodium sulfate fractions of F II (SS_1 to SS_6).

extensive study of this phenomenon, in which a wider range of reaction volumes was evaluated, is shown in Fig. 8. The essential difference between fraction SS_2 and whole F II was the greater amount of non-aggregated 7S gamma globulin in the latter. The addition of large amounts of 7S gamma globulin to fraction SS_2, *i.e.* in essence a reconstitution of whole F II, was associated with a return of the volume effect. Thus, in addition to the failure of 7S gamma globulin to induce precipitation with rheumatoid serum, in high concentration it appeared to exert an inhibitory effect on the precipitin reaction.

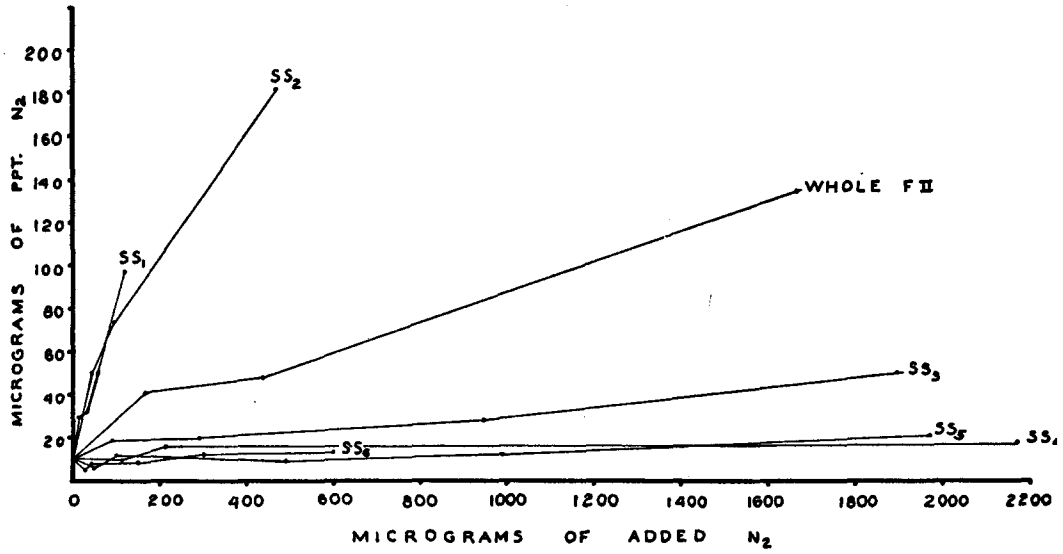

FIG. 5. Precipitin curves of whole F II and sodium sulfate fractions of F II (SS_1 to SS_6) added to 0.25 ml. rheumatoid sera (H.P.).

Aside from the effect of 7S gamma globulin in small reaction volumes, an inhibitory property of the non-aggregated material could be demonstrated in higher dilutions in which there was no volume effect. This was apparent from the shapes of precipitin curves (see Fig. 9) when equal amounts of fraction SS_2, one diluted in buffered saline and one diluted in gamma globulin prepared by electrophoresis convection, were added to a rheumatoid serum. The shape of the gamma globulin diluent curve resembled the precipitin pattern of whole F II. Dilution of fraction SS_2 in concentrations of human serum albumin equivalent to that of the electrophoresis-convection gamma globulin did not result in inhibition of precipitate formation.

The possibility of performing precipitin studies in small reaction volumes with fractions SS_1 and SS_2 permitted ultracentrifuge studies on the supernatants of the precipitin reactions. Fig. 10 illustrates a precipitin study in

which 0.6 cc. aliquots of a rheumatoid serum were allowed to react with varying quantities of a reprecipitated fraction SS_1 (the latter with an approximate s_{20} value of 40 was essentially free of more slowly sedimenting material). The ultracentrifuge patterns above the precipitin points characterize the supernatants at each point. The supernatant after removal of spontaneous precipitate (lower left pattern) showed a large amount of a rapidly sedimenting material. This component, which was demonstrated in about one-third of rheumatoid sera (notably sera with high serological titers) by Franklin *et al.*, had

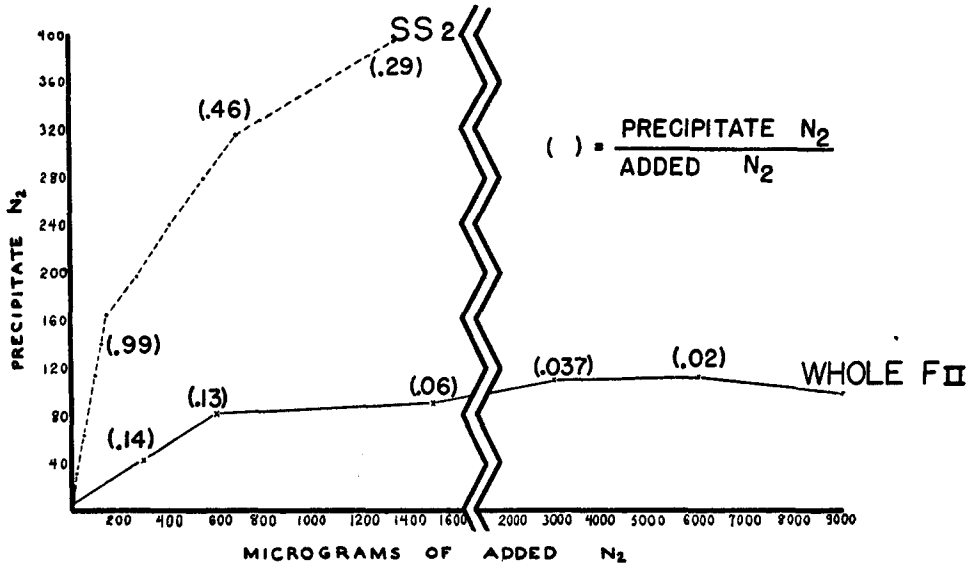

FIG. 6. Precipitin study. Comparison of whole F II and sodium sulfate fraction 2 (SS_2) plus 0.25 ml. rheumatoid serum (W.E.).

an s_{20} value of 22 (7, 8). As the quantity of added fraction SS_1 was progressively increased, the precipitate yield correspondingly increased and the 22 S component diminished until at the last point it was no longer detectable. No significant change was noted in the slower 19S component. The decrease in the F II tanned sheep cell agglutination titer of the supernatants paralleled the disappearance of the 22S material.

3. F II Tanned Sheep Cell Inhibition.—This procedure, outlined under Materials and Methods, measured the degree to which a given material could inhibit the F II tanned cell agglutinating activity of a serologically positive rheumatoid serum. The inhibitory activities of whole F II and the sodium sulfate fractions are summarized in Fig. 11. Fractions SS_1 and SS_2 inhibited in very low concentration. Whole F II and fractions SS_3 to SS_6 inhibited only

with much larger amounts. The concentrations of fractions SS_4 to SS_6 required for complete inhibition were approximately 200 times the inhibiting concentrations of fractions SS_1 and SS_2.

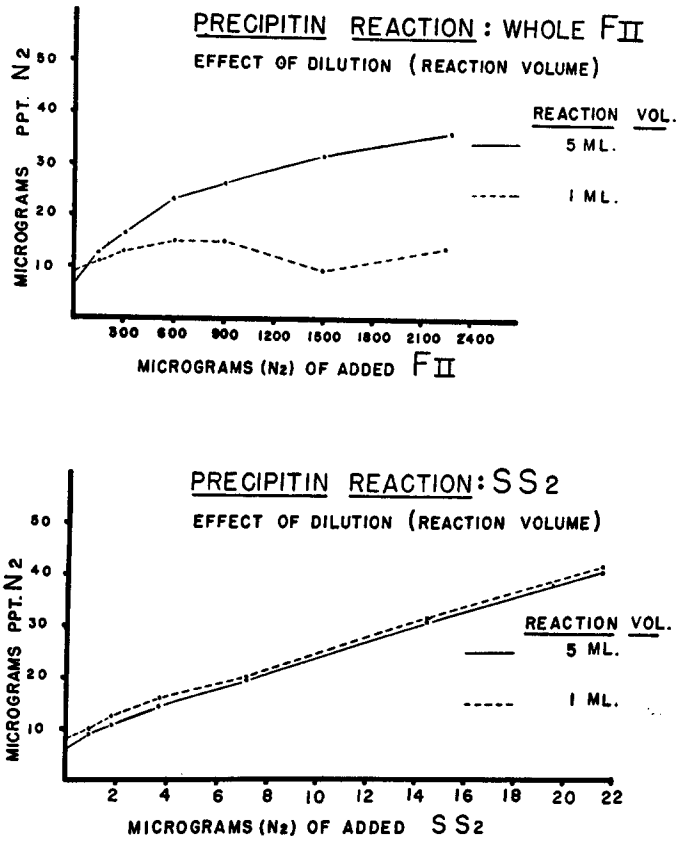

FIG. 7. Demonstration of the volume effect (relation of increased precipitate yield relative to the degree of dilution) in whole F II precipitin studies. Comparison with SS_2 precipitin reaction which does not show the volume effect.

4. Tanned Sheep Cell Agglutination with Sodium Sulfate Fractions of F II as Sensitizing Materials.—This study was performed in a manner identical with the F II tanned sheep cell agglutination test (Heller) with the exception that sodium sulfate fractions were used as coating (sensitizing) materials. Tanned sheep erythrocytes were incubated with varying amounts of the fractions and then tested with a reference serum (routine F II tanned sheep cell titer 1:3200). The results are summarized in Fig. 12. Relatively low concentrations of fractions SS_1 and SS_2 were required in achieving a degree of sen-

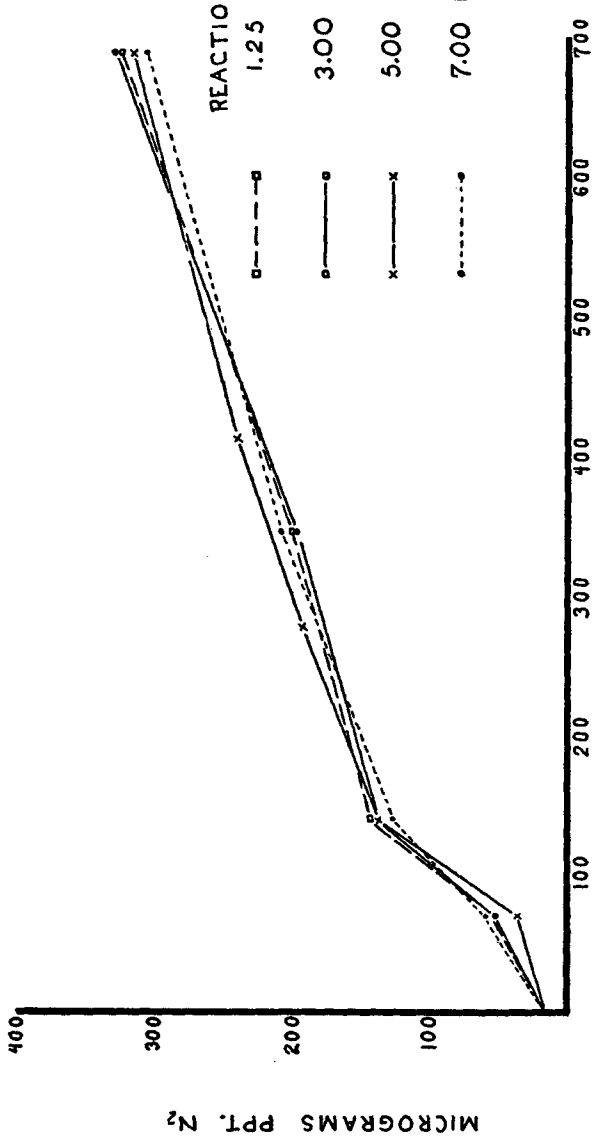

FIG. 8. Effect of dilution (reaction volume) on precipitin reaction with sodium sulfate fraction 2 (SS_2) + 0.25 ml. rheumatoid serum (W.E.). The absence of the volume effect is shown.

sitization equal to the routine F II agglutination test; *i.e.*, agglutination at a 1:3200 dilution of the reference rheumatoid serum. High concentrations of fraction SS_3 gave incomplete sensitization and fractions SS_4 to SS_6 gave no sensitization in high concentrations.

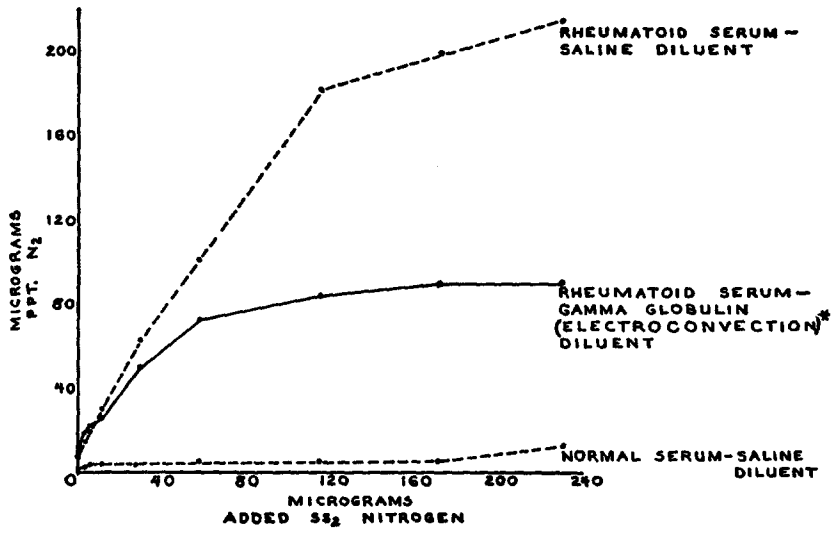

FIG. 9. Precipitin study: Effect of non-reactive gamma globulin (electroconvection) on precipitate yield from sodium sulfate fraction$_2$ (SS_2) plus rheumatoid serum. SS_2 twice reprecipitated—free of detectable 7S component. The inhibitory effect of non-precipitating gamma globulin is shown. The ratio of non-precipitating gamma globulin nitrogen to fraction SS_2 nitrogen in the gamma globulin diluent study was 40/1.

* Concentration of gamma globulin—2.33 mg./cc.

DISCUSSION

The material in F II (gamma globulin) which reacts with "rheumatoid factor" in the F II precipitin test and the F II tanned sheep cell agglutination test could be greatly concentrated by sodium sulfate fractionation. The concentrated material, *i.e.* the "reactant," consisted of polydispersed molecular aggregates of gamma globulin with sedimentation constants greater than 7. Gamma globulin preparations, in which aggregated materials (s_{20} greater than 7) were not detectable, reacted little or not at all in the precipitin reaction and were relatively ineffective in inhibiting the F II tanned sheep cell agglutination test or in sensitizing tanned sheep erythrocytes to the agglutinating action of a serologically positive serum. The converse of the above statement applied to the preparations of aggregated gamma globulin; *i.e.*, such materials

reacted strongly in the precipitin reaction and in low concentration inhibited F II tanned sheep cell agglutination and sensitized tanned erythrocytes.

A comparison of precipitin character of aggregated gamma globulin (fractions SS_1 and SS_2) and whole F II revealed striking differences: (1) With refractionated preparations of SS_1 and SS_2 that were relatively free of non-aggregated 7S gamma globulin, as shown in Fig. 10, the ratio of precipitate nitrogen to nitrogen added was as high as 2.0. The same ratio for whole F II preparations varied between 0.05 and 0.2. (2) Fig. 6 demonstrates the differ-

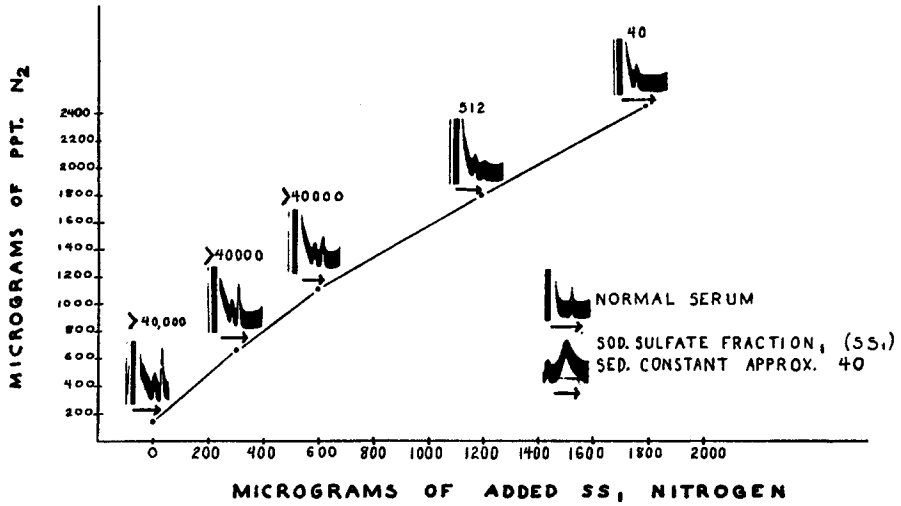

FIG. 10. Precipitin study with reprecipitated fraction SS_1 plus 0.6 ml. rheumatoid serum (M. B.). Ultracentrifugal characterization and F II tanned sheep cell agglutination titers of the supernatants. The ultracentrifuge patterns of supernatants are 48 minute exposures at 47,660 R.P.M. The heavier component (tallest peak) is the 22S complex. The smaller component is 19S.

ence in shape of precipitin curves with aggregated gamma globulin (SS_2) *versus* whole F II. The precipitin curves of fractions SS_1 and SS_2, under the conditions studied, continued to rise without reaching a plateau or decline (properties which whole F II precipitin studies exhibit). These precipitin properties of whole F II are probably due to the inhibitory effect of non-aggregated gamma globulin (7S) on precipitate formation (see Fig. 9). The observation of Epstein that precipitates formed in the F II precipitin reaction could be dissolved in a large excess of gamma globulin supports this view (17). The volume effect, *i.e.* the relation of increased precipitate yield to increased reaction volume, which is characteristic of whole F II precipitin studies, was not noted with aggregated materials (see Fig. 7). The inhibition of precipitate formation in whole F II precipitin studies in low reaction volumes could also

be attributed to the inhibitory property of non-aggregated gamma globulin (7S), since the addition of large amounts of 7S material to fractions SS_1 or SS_2 reproduced the volume effect.

The low concentrations of aggregated gamma globulin (fractions SS_1 and SS_2) required for sensitization of tanned erythrocytes and the ineffectiveness of high concentrations of non-aggregated materials ($SS_{4 \text{ to } 6}$) strongly suggest that the aggregated material is the basis for sensitization in the F II tanned cell agglutination system. In the routine F II tanned cell test, whatever aggregation is already present in F II is augmented by heating to 56°C. for 30 min-

FRACTIONS	INHIBITING CONCENTRATION
WHOLE FII	.92
SS_1	.04
SS_2	.06
SS_3	1.7
SS_4	7.8
SS_5	9.5
SS_6	10.75

FIG. 11. Concentrations of whole F II and sodium sulfate fractions (SS) of F II capable of completely inhibiting the F II tanned cell agglutinating activity of a diluted rheumatoid serum. Dilution of the rheumatoid serum was such that the F II tanned cell agglutinating titer of the diluted material was 1:12. Concentrations expressed as micrograms of nitrogen per 0.5 ml.

utes (inactivation of complement).[4] Heating a non-precipitating preparation of gamma globulin under these conditions produced aggregation as evidenced by development of opalescence and reactivity in the precipitin test. Since most of the standard serological tests for rheumatoid arthritis involve the use of either F II or materials which have been heated, aggregated gamma globulin *may* be the *sine qua non* for sensitization in all the serological systems. One

[4] Commercial production of pooled human fraction II involves the use of temperatures greater than 37°C. during 2 steps: (1) plasma is heated to 50°C. for 48 hours prior to fractionation, and (2) lyophilized F II is subjected to a temperature of 60°C. for 24 hours prior to its distribution for clinical use (21). (The latter step does not apply to F II that is sold for non-clinical use.) The above probably accounts for the failure of non-commercial preparations of F II to react in the precipitin test (22), as opposed to the constant reactivity of commercial sources of F II.

significant difference between the systems herein studied (F II tanned sheep cell agglutination and F II precipitin reactions) and most other rheumatoid serological systems (sensitized sheep cell test, Rh-sensitized system (18, 19),

FRACTION	CONC. (MICROGM. NITROGEN PER 2 CC.)	AGGLUT. TITER OF RHEUMATOID SERA POOL	LOWEST CONC. WHICH SENSITIZES TO SAME DEGREE AS ROUTINE F II TANNED CELL TEST (HELLER)
WHOLE F II	745	6400	372.0
	372	3200	
	186	1600	
	93	1600	
	46	1600	
SS_1	35	6400	17.5
	17.5	3200	
	8.8	1600	
	4.4	200	
	2.2	100	
SS_2	1440	>51200	90.0
	720	>51200	
	360	51200	
	180	25600	
	90	3200	
SS_3	2460	1600	SENSITIZATION NOT COMPLETE AT 2460 MICROGM. $N_2/2^{cc}$
	1230	1600	
	615	1600	
	308	800	
	154	200	
SS_4	1780	0	NO SENSITIZATION AT 1780 MICROGM. $N_2/2^{cc}$
	890	0	
	445	0	
	222	0	
	111	0	
SS_5	900	0	NO SENSITIZATION AT 900 MICROGM. $N_2/2^{cc}$
	450	0	
	225	0	
	112.5	0	
	56	0	
SS_6	248	0	NO SENSITIZATION AT 248 MICROGM. $N_2/2^{cc}$
	124	0	
	62	0	
	31	0	
	15.5	0	

FIG. 12. Results of coating tanned sheep erythrocytes with varying concentrations of whole F II and sodium sulfate fractions of F II. Determination of agglutinating titer of a standard rheumatoid sera pool. (Routine F II tanned cell agglutination titer (Heller) 1:3200). The titers are expressed as the reciprocal of serum dilutions.

and the reaction of "rheumatoid factor" with immune precipitates (20)) is that sensitization in the latter test systems involves an immune reaction; *i.e.*, a combination of an antigen and its specific antibody. The sheep erythrocytes in the F II tanned sheep cell test are coated with gamma globulin, but not *via* an immunological reaction. The "rheumatoid factor" may react primarily with antigen-antibody complexes. The aggregation of gamma globulin which

is essential for sensitization in the F II precipitin and the F II tanned sheep cell tests may simulate the aggregation of antibody gamma globulin that results from an immune reaction.

The foregoing information has permitted speculation as to the molecular basis of the rheumatoid serological reactions. Reaction I in Fig. 13 represents the soluble complexing of "rheumatoid factor" (19S) with non-aggregated gamma globulin (7S) into the 22S component described by Franklin *et al.* (7, 8). Reaction II illustrates the combination of "rheumatoid factor" with aggregated gamma globulin. Evidence in this report suggests that reaction II is the basis for the F II precipitin and F II tanned sheep cell agglutination systems. The 22S material under certain conditions dissociates into 19S and

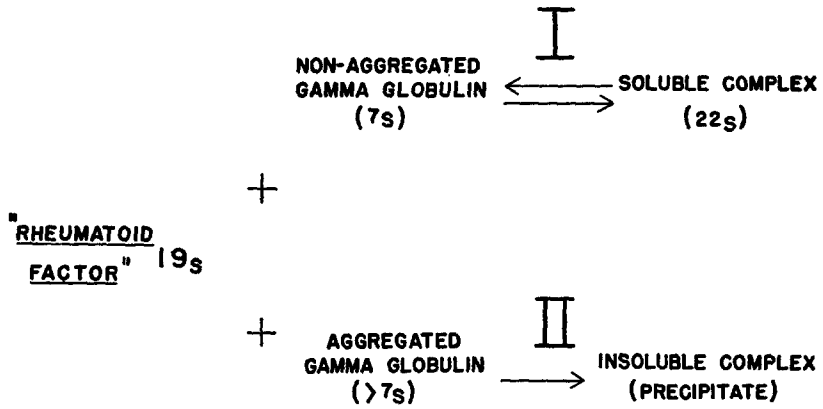

FIG. 13. Schematic hypothesis for molecular basis of the rheumatoid serological reactions.

7S components. There is not direct proof that this phenomenon is reversible; *i.e.*, reassociation of 19S and 7S molecules into the 22S complex. The observation, however, in the present study that large amounts of 7S gamma globulin inhibited reaction II is indirect evidence that reassociation of 7S and 19S components does occur.

Considerable attention has been devoted to the phenomenon of inhibition of sensitized sheep cell agglutination by normal sera (4, 23). Ziff *et al.* developed a technique of testing serum euglobulin both for agglutination of sensitized sheep cells and inhibition of sensitized sheep cell agglutination. A serum is considered positive if its euglobulin agglutinates in the sensitized sheep cell system, or, if failing that, it fails to inhibit a serologically positive serum. Since the serum from which the euglobulin is obtained is heated (56°C.) to inactivate complement, some aggregation of gamma globulin probably occurs. (Heating to that extent induced precipitin reactivity in a previously nonreactive preparation of gamma globulin.) The aggregated material formed in

the euglobulin of a serum with a low concentration of "rheumatoid factor" might then rapidly precipitate with the latter, making the agglutination procedure negative. Because the aggregated gamma globulin is also removed—which would not occur with a non-rheumatoid euglobulin—the rheumatoid euglobulin fails to inhibit the agglutinating property of a serologically positive serum. Since, in the present study, inhibition of the F II tanned sheep cell agglutination by non-aggregated gamma globulin (7S) was of very low magnitude as compared to aggregated materials, the phenomenon of sensitized sheep cell inhibition is most likely due to the aggregated gamma globulin that is formed by the test procedure.

The "rheumatoid factor" fulfills some of the criteria of an antibody. Some known antibodies are present in the 19S fraction of gamma globulin (15, 24, 25). Prior interpretation of the F II precipitin reaction suggested that the inhibition of precipitate formation with an excess of F II might be analogous to the zone of antigen excess in established antigen-antibody systems. The present data support the concept that the F II precipitin inhibition by an excess of F II is the result of the solubilizing effect of non-aggregated gamma globulin. (The great abundance of the non-aggregated material (7S) in whole F II relative to aggregated components is indicated by the failure to detect the latter with analytical untracentrifugation.) The failure of precipitin studies, using fractions SS_1 and SS_2 to demonstrate a zone of precipitate inhibition with "antigen" excess, is not inconsistent with the reaction being antigen-antibody in nature. The precipitin character of tobacco mosaic virus (a macromolecular material) with rabbit antibody against the virus is similar to the aggregated gamma globulin precipitin studies; *i.e.*, the precipitin curves are similar in shape and both show continued precipitate formation beyond the equivalence point (the point at which "antibody" is no longer detectable in the supernatants) (26, 27).

Information is not available at present to establish clearly the reaction of "rheumatoid factor" with the "reactant" as an antigen-antibody combination rather than some other type of protein-protein interaction. Although some techniques of immunochemical study suggest an antibody role for the "rheumatoid factor," the crucial demonstration that gamma globulin in some form serves as an antigenic stimulus for production of the "rheumatoid factor" is lacking. Since the forces that are applied *in vitro* to produce molecular aggregation of gamma globulin probably represent protein denaturation, the physiological significance of the aggregated material is unknown. Indirect evidence that aggregated gamma globulin may exist in the absence of denaturation is offered by the observation that some rheumatoid sera precipitate spontaneously in the cold (28). The spontaneous precipitates when dissolved in an acid buffer have sedimentation properties that are similar to those of gamma globulin–induced precipitates (29). "Rheumatoid factor" can be eluted from

both spontaneous precipitates and gamma globulin–induced precipitates with 15 per cent NaCl (30).

SUMMARY

The material in gamma globulin ("reactant") which reacts with rheumatoid sera in the F II precipitin and F II tanned sheep cell agglutination tests was concentrated by precipitation with sodium sulfate. The concentrated "reactant" appeared to consist of polydispersed molecular aggregates of gamma globulin with s_{20} constants as high as 40, as well as the previously described inhomogeneous aggregated material.

Aggregated gamma globulin precipitated readily with most rheumatoid sera regardless of the reaction volume, and in low concentration inhibited the F II tanned sheep cell agglutination reaction and sensitized tanned erythrocytes to the agglutinating action of a positive rheumatoid serum. On the other hand, non-aggregated gamma globulin (7S) did not precipitate with rheumatoid sera and in low concentration did not inhibit the F II tanned sheep cell agglutination reaction, or sensitize tanned erythrocytes.

Non-aggregated gamma globulin in large excess inhibited the precipitin reaction of aggregated gamma globulin with "rheumatoid factor," and accounted for the characteristic shape of the whole F II precipitin curves and the volume effect described by Vaughan (relation of increased precipitate yield relative to the reaction volume in whole F II precipitin studies).

In serological systems other than the F II tanned sheep cell and F II precipitin reactions, the sensitization involves an antigen-antibody combination; *i.e.*, sheep erythrocyte plus hemolysin in the sensitized sheep cell test, and egg albumin plus anti egg albumin in the absorption experiments with specific precipitates. The aggregation of gamma globulin that was essential for sensitization in the F II tanned sheep cell and F II precipitin tests may have simulated the aggregation of antibody gamma globulin that occurs with antigen-antibody union.

The present information has been incorporated into a schematic hypothesis for the basis of the rheumatoid serological reactions.

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