SIII. The slight increase in skin reactions with the guinea pigs on D19 is at present unexplainable.

Summary. The lack of antigenicity of purified pneumococcal polysaccharide and dextran in guinea pigs has been demonstrated. Both the methods used for immunization and detection of antibody have been shown to be quite good for the other systems.

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Purification of a Rheumatoid Factor.* (24357)

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The serological reactions of the rheumatoid factor have been studied extensively not only for the promise they hold as a diagnostic tool, but also for their possible bearing on the etiology of rheumatoid arthritis. A critical review of past work has appeared recently(1). The rheumatoid factor is a macroglobulin with a sedimentation coefficient of $s_{w,20} =$ 19 S; in many instances it circulates in the blood as a complex ($s_{w,20} =$ 22 S) containing other gamma globulin(11). The rheumatoid factor, furthermore, contains 2 serologically distinct and separate components(2,3,4). While both components react with aggregated gamma globulin(5,6), only one seems involved in adsorption onto certain antigen-antibody systems(3). Such systems form the basis of the various sensitized erythrocyte(7,8) and bacterial agglutination reactions(9). A number of investigations aimed at isolation of the rheumatoid factors have been reported recently(4,10,12,13,14). A new simple method is reported herewith, which avoids the compli-

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cations arising from use of an antigenic eluent (10), extremes of pH(12) or high concentrations of urea(4,12). The method extends and applies recent studies on pH dependence of the sensitized sheep cell reaction(15). It was shown that optimal conditions for the reaction lie between pH 6 and 8.5, while inhibition occurs below pH 5.8. In applying this knowledge, the agglutination of sensitized sheep erythrocytes due to a constituent of serum of individuals with rheumatoid arthritis was reversed by adjusting the test suspension to pH 5.5. Dissociation of the agglutination factor from the sheep erythrocyte anti-sheep erythrocyte complex was observed. The decantate of such a test suspension contained the agglutination factor, whereas the sedimented cells on readjustment of pH could be reemployed in further agglutination tests.

Materials and methods. Complications arising from the use of intact sheep erythrocytes were overcome by use of stroma. Stroma preparations, obtained from pooled sheep blood, were sensitized with varying amounts of rabbit anti-sheep serum. The optimal sensitizing dose was determined for each batch of stroma by testing its affinity for the agglutination factor. The optimal sensitizing dose in most experiments was found to exceed the basic agglutination titer by a factor of 100. The volume of packed sensitized stroma required for the complete absorption of the factor agglutinating sensitized sheep erythrocytes depended on the titer of the serum to be processed. Individual serum samples, previously stored in a freezer from one to three months, were used. Purification of serum samples was followed by the sensitized sheep cell test, using 1/20 of the basic agglutination titer of the rabbit anti-sheep serum as the sensitizing dose for a 0.25% suspension of sheep erythrocytes(7), and by the latex fixation test(16). Heterophile antibodies were tested by a 0.25% sheep erythrocyte suspension. Protein analyses were performed by automatically recorded ultraviolet absorption spectrophotometry. The difference in extinction coefficients between 278 mμ and 320 mμ served as index of the protein concentration. A gamma globulin reference standard was used. Preparative as well as analytical ultracentrifugation was carried out in a Spinco Ultracentrifuge, Model E. A preparative rotor "K", with tubes of 38.5 ml and 6.5 ml capacity respectively, was used. Analyses were performed at 47,660 rpm, in a cell equipped with a double sector centerpiece for simultaneous determination of the sedimentation characteristics of solution and solvent. Determinations of the sedimentation coefficients, when possible, were made at different concentration by observation of the sedimentation velocity. Homogeneity of the macroglobulin was determined by calculating apparent diffusion constants (D_{app}) from measurements of area and maximal ordinates of boundaries at different time intervals as suggested by Schachman(17). As insufficient amounts of material were available for elecrophoretic analyses by a moving boundary method, paper electrophoresis was resorted to. An E. C. electrophoresis apparatus, Whatman Filter Paper 3MM, veronal buffer (0.05 Γ/2, pH 8.6) and a field strength of 500 V over a 5 hour period were used. Serum samples were diluted 10-fold with distilled water and adjusted to pH 6.4. After storage overnight in the cold, the precipitated material was spun off and resuspended in 0.15 M phosphate buffer of pH 7.0. The solution was exposed to freshly sensitized sheep erythrocyte stroma for one hour. The amount of stroma required for complete absorption of the agglutination factor depended on the stroma preparation as well as on the titer of the serum sample. The stroma-agglutination factor complex was washed at least 6 times with saline and finally suspended in aliquots of 0.15 M phosphate buffer of pH 5.5, with stirring for one hour. Following centrifugation, this procedure was repeated until no further agglutination factor appeared in the supernatant. The combined supernatants were spun in the ultracentrifuge at 140,000 x g for 16 hours. The resulting pellets were dissolved in 0.15 M phosphate buffer of pH 5.5, and subjected to differential ultracentrifugation in a 25% sucrose-saline density gradient. After 3 hours of centrifugation at 140,000 x g the agglutination factor was found in fractions of highest sucrose concentration. The fractions were dialyzed against 0.15 M phosphate buffer of pH 5.5.

TABLE I. Progress of Purification of the Sensitized Sheep Erythrocyte Agglutination Factor.

	Specific activity*	
	Sensitized sheep erythrocyte agglutination test	Latex gamma globulin fixation test
Initial serum sample (I.A.)	140	70
Sample after precipitation at pH 6.4, low ionic strength	3,200	3,200
Sample after removal of the Agglutination Factor	0	950
Agglutination Factor released from sensitized sheep erythrocyte stroma after exposure to pH 5.5	8,000	4,500
Agglutination Factor after repeated differential ultracentrifugation	80,000	40,000

* Titer/mg protein.

The differential ultracentrifugation was repeated until a solution devoid of low molecular weight contaminants was obtained.

Results. Table I is reduced protocol of a successful purification experiment. Considerable purification was obtained by the initial precipitation from a solution at low ionic strength and pH 6.4. The new method, however, afforded selective separation of the material responsible for agglutination of sensitized sheep erythrocytes from other euglobulins, including a material which reacted with aggregated gamma globulin but not with sensitized sheep erythrocytes. Elution of the sensitized sheep stroma complex with 0.15 M phosphate buffer of pH 5.5 resulted in a good recovery of the agglutination factor. Analytical ultracentrifugation of the eluate revealed some impurities of low molecular weight ostensibly derived from soluble stroma. The impurities were removed by repeated differential ultracentrifugation. Large manipulative losses, mainly due to precipitation of material during dialysis for the removal of sucrose, occurred. Dialysis at neutral or at slightly alkaline pH accelerated the formation of insoluble precipitates. The specific activities of the agglutination factor were expressed in titers obtained from the assay of 1 mg of protein. As can be seen, a protein in concentration of 0.013 μg still agglutinated sensitized sheep erythrocytes, and 0.026 μg still fixed gamma globulin-latex particles. Heterophile antibody was absent. Sedimentation patterns of 2 preparations are shown in Fig. 1. Table II provides a summary of the results

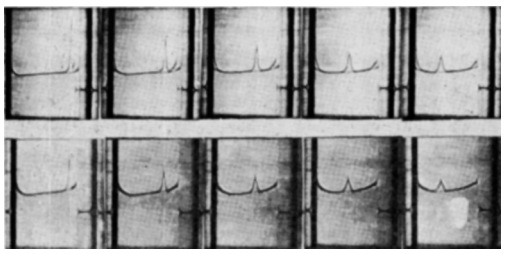

FIG. 1. Sedimentation diagrams of preparations (I.A.) and (W.S. I). 47,660 rpm. Time interval: 8 min. Phase plate angles 48.5 and 39.5 deg.

TABLE II. Summary of Results of Purification Experiments of Five Serum Samples.

Serum sample	Specific activities: Sensitized sheep cell aggl. test	Specific activities: Latex γ globulin fixation	Paper electrophoresis
	× 1000	× 1000	
I.A.	80	40	—
W.S. I	60	30	—
W.S. II	12	8	—
M.W.	18	48	Gamma globulin
M.P.	13	50	*Idem*

obtained with other serum samples. The calculated sedimentation coefficients of the most highly purified preparations (I.A., W.S. I) were $s_{obs} = 20.2$ s, corrected to $s_{w,20} = 18.2$ s. The diffusion constant, obtained from sedimentation pattern (I.A.) was $D_{app} = 1.94 \times 10^{-7}$ cm^{2}sec^{-1}. Details of the calculation appear in Table III.

Control experiments with two sera of individuals without connective tissue disease failed to yield a similar macroglobulin.

Discussion. The use of sensitized sheep erythrocyte stroma for the absorption of the agglutination factor has enabled preparation of a highly purified and reactive macroglobu-

TABLE III. Calculation of an Apparent Diffusion Constant (D_{app}) from a Sedimentation Diagram (I.A.).

Time (t), sec.	$1-\omega^2st$	A, mm^2	H, mm	D_{app}, $cm^2\ sec^{-1}$
480	.978	3.63	10.35	1.98×10^{-7}
960	.955	3.30	6.45	2.08 "
1,440	.933	2.70	4.40	1.94 "
1,880	.911	2.40	3.35	1.94 "
2,360	.889	2.26	2.80	1.91 "

$$D_{app} = \frac{1}{4\pi t}\left(\frac{A}{H}\right)^2 (1-\omega^2 st),$$

where s = Sedimentation coefficient (20.2×10^{-13} sec.)
ω = Revolutions/second $\times 2\pi$ (radians)
A = Area under peak
H = Height of maximal ordinate of peak

lin. Its specific activity greatly exceeds the values previously reported by others(4,6). Tentative evidence for the homogeneity of one preparation rests on the close agreement of the values of D_{app} at different time intervals, as detailed in Table III. On substitution of D_{app} into an equation combining sedimentation and diffusion constants, an estimated molecular weight of the agglutination factor could be calculated. An assumed partial specific volume $\bar{V}$ of 0.725 was needed for the estimate:

$$MW = \frac{R\,T\,s}{D\,(1-\bar{V})} = \frac{8.13 \times 10^{-7} \times 299 \times 20.2 \times 10^{-13}}{1.94 \times 10^{-7}\,(1-0.725)}$$

$$= 950{,}000$$

The agglutination factor was separated from a second macroglobulin of rheumatoid arthritis, which although reacting with gamma globulin (latex fixation), failed to agglutinate sensitized sheep erythrocytes. Isolation procedures based on the precipitation of the rheumatoid factors by gamma globulin(6) result in the simultaneous precipitation of both proteins and thus render a mixture of macroglobulins. Recent reports indicate, however, a successful resolution of this mixture on a DEAE cellulose ion exchange column(4). Barring structural changes due to concentrated urea, the material purified on this column and the macroglobulin described in this paper seem to be identical.

Based on calculations of specific activity, the agglutination factor occurs in concentrations ranging from 0.1 mg to 20 mg per cent in sera of individuals with positive sensitized sheep cell titers.

Summary. A macroglobulin was purified from sera of individuals with rheumatoid arthritis which agglutinated sensitized sheep erythrocytes and fixed gamma globulin-latex particle suspensions. Tentative evidence for the homogeneity of a preparation was furnished. The isolated macroglobulin is a component of the rheumatoid factor. The latter is a mixture of closely related macroglobulins.

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