ferring to the inhibitor of agglutination of sensitized sheep cells by rheumatoid sera and assigned this activity to some component of the FII fraction of serum. Based on this observation, tanned sheep erythrocytes were coated with γ-globulin and employed for detection of the agglutinating factor in rheumatoid sera. The test for agglutination-inhibitor as devised by Ziff *et al.*(7) is associated with the euglobulin fraction of serum. It was postulated that all sera probably contain inhibitor, but that it cannot be readily demonstrated in the euglobulin fraction of rheumatoid sera because of neutralization by the rheumatoid factor. Albumin was found to be free of inhibitive properties by both groups of workers.

In the present report the influence of serum albumin in the latex-fixation technic most probably is a function of its protective colloid properties and may reflect certain, as yet unexplained, aspects of the test.

Summary. 1. Thirty-two of 94 rheumatoid sera, positive by the latex-fixation test when diluted 1:20, also reacted in the undiluted state. 2. Sodium sulfate precipitation of the remaining 62 sera permitted positive demonstrations with the reconstituted globulins. 3. No alteration in titer was associated with the precipitation procedure. 4 Electrophoretic patterns revealed that the reactive undiluted sera manifested a reduced albumin content.

1. Singer, J. M., Plotz, C. M., *Am. J. Med.*, 1956, v21, 888.

2. Plotz, C. M., Singer, J. M., *ibid.*, 1956, v21, 893.

3. Rheins, M. S., McCoy, F. W., Buehler, E. V., Burrell, R. G., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 67.

4. Rheins, M. S., McCoy, F. W., Burrell, R. G., Buehler, E. V., *J. Lab. Clin. Med.*, 1957, v50, 113.

5. Thurston, J. R., Rheins, M. S., Buehler, E. V., *ibid.*, 1957, v49, 647.

6. Heller, G., Jacobson, A. S., Kolodny, M. H., Kammerer, W. H., *J. Immunol.*, 1954, v72, 66.

7. Ziff, M., Brown, P., Lospalluto, J., Badin, J., McEwin, C., *Am. J. Med.*, 1956, v20, 500.

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Biological Activity of Soluble Antigen-Antibody Complexes I. Skin Reactive Properties.* (23829)

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As a result of some preliminary studies made in conjunction with an investigation on the effect of soluble complexes on smooth muscle(1), it became obvious that soluble antigen-antibody complexes formed in excess antigen also produced a marked increase in permeability of capillaries in guinea pig skin. This was shown by the fact that when a small amount of soluble antigen-antibody complex was injected into the skin of normal guinea pigs which had previously received an intravenous injection of Evans Blue, the area of injection became blue. The following paper presents a more detailed investigation of this phenomenon. The first work involved fractionation of antiserums by starch electrophoresis to determine if the antibody involved was a precipitating gamma globulin. The second study was made on normal and immune serums without the addition of any antigen when it became apparent that immune as well as normal serums contained a skin-irritating protein component.

Materials and methods. Antiserums: In the present investigation, only Armour's bovine serum albumin (BSA) and rabbit antiserum were studied. Three different pools (1, 2 and 3) of antiserum were used but immunization was the same in all rabbits. The schedule consisted of 10 mg of BSA intravenously 3 times a week for 4 weeks, then bleeding 7 days after the last injection. Serum from each rabbit was titrated by qualitative

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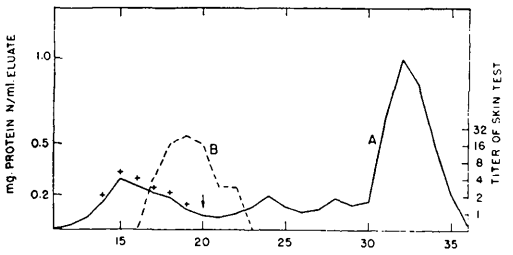

FIG. 1. Starch electrophoretic pattern of rabbit anti-BSA No. 1. Curve A represents conc. of protein N in eluates. Curve B indicates titer of skin test. Plus indicates presence of precipitating antibody. Arrow indicates the origin.

tests for content of precipitating antibody and those having approximately the same titer were pooled and the pools stored at −40°C. The amount of precipitable antibody N per ml in the 3 different pools was, No. 1 = 650 μg, No. 2 = 145 μg, and No. 3 = 369 μg. *Zonal electrophoresis* on starch blocks was carried out in the usual manner using barbital buffer pH 8.6, μ = 0.10. Serum samples of about 6 ml were allowed to separate for about 19 hours at 60 MA. The starch block was then cut into 1.0 cm segments and each segment placed in a 50 ml centrifuge tube and eluted with 9 ml of saline. The amount of protein in each eluate was determined by biuret analysis(2). *Skin reactions* were all made on guinea pigs by a modification of the method described by Ovary(3). Guinea pigs weighing about 500 g were given intravenously 1.5 ml of a 0.5% solution of Evans Blue per kg body weight. Test solutions consisting of 0.1 ml were then immediately injected intracutaneously in the shaved lumbar back surface of the "blued" animals. Control sites consisted of 0.1 ml of pyrogen-free saline and 0.1 ml pyrogen-free saline containing 0.3 μg of histamine phosphate. A positive reaction became quite apparent in 2-4 minutes and reached maximum in 20-30 minutes. Animals were sacrificed 30 minutes after injection and the test area of the skin removed. The reactions were graded by appearance and average diameter of blueing according to the following scheme: negative, less than 5 mm; 1 plus, 5 to 7 mm; 2 plus, 7 to 10 mm; 3 plus, more than 10 mm and deep color.

Results. A. *Soluble antigen-antibody complexes.* The first studies were made on whole serums No. 1 and 2. Skin tests were made on the supernatants obtained from precipitin reactions. The precipitin reactions were set up in the usual manner in which equal volumes of various 2-fold dilutions of antigen and undiluted antiserum were mixed, allowed to stand for 48 hours at 4°C and the supernatants obtained by centrifugation. Each supernatant was then tested in various dilutions (from undiluted to 1:128). Supernatants from tests made with serum No. 2 showed maximum skin reaction in slight antigen excess (1:64) and less at equivalence and antibody excess (1:16 to 1:32). With serum No. 1 there was a slight tendency for skin reactions to be strongest in supernates from the equivalence region and slight antigen excess, but fairly severe skin reactions were obtained in dilutions of 1:64 over the entire range of antigen-antiserum tested. It became apparent upon testing the antigen and the antiserums separately that the antiserum alone contained an irritating component.

In view of this finding, the following experiment was carried out to determine whether the irritating substance could be separated from precipitating antibody by electrophoretic fractionation on starch. Six ml samples of antiserums No. 1 and 2 were allowed to migrate on a starch block for 19 hours, then cut into 1.0 cm segments and the protein eluted from each segment. Each eluate was tested for precipitins by ring test and for skin activity. The results are given in Fig. 1 and 2. Some over-lapping occurred in the region of "fast gamma" but a few eluates in "slow

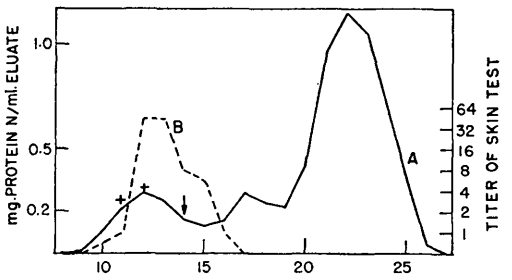

FIG. 2. Starch electrophoretic pattern of rabbit anti-BSA No. 2. Curve A represents conc. of protein N in eluates. Curve B indicates titer of skin test. Plus indicates presence of precipitating antibody. Arrow indicates the origin.

TABLE I. Activity of Supernates from Quantitative Precipitin Analyses.

Antigen N added	Total N precipitated	Antibody N precipitated	Tests on supernate: Presence of Ag or Ab	Tests on supernate: Skin test
	μg			
1 ml, slow γ globulin fraction from antiserum No. 2 .25 ml of antigen were used				
80	0	0	Excess antigen	+++†
40	7.6		*Idem*	+++
20	41.7		"	+++
10	63.4	ca 53.4	Trace antigen	+++
5	51.9	46.9	Neither	—
2.5	30.3	27.8	Trace antibody	—
1.25	14.8	13.5	Excess "	—
0	0	0	Antibody	—
2 ml, slow γ globulin fraction from antiserum No. 3 .5 ml of antigen were used				
160	1.9		Excess antigen	1:4*
80	9.5		*Idem*	1:8
40	52.6		"	1:4
20	89.6	ca 69.6	Trace antigen	1
10	87.4	77.4	Neither	—
5	52.5	47.5	"	—
2.5	26.8	24.3	Trace antibody	—
0	0	0	Antibody	—

* Minimum conc. of supernate required to produce skin reaction.

† Similar reactions were obtained by 1:2 dilution.

BSA (320 μg N/ml) produced no reaction.

gamma" of serum No. 1 gave only precipitin activity and a few in the area between gamma and beta gave only skin activity. The low titer serum No. 2 showed over-lapping in the slow gamma area, but skin activity and no precipitin in the fast gamma. The three eluates (No. 14, 15 and 16) of serum No. 1 were then combined and antigen added in varying dilutions. The results of this test are given in Table I. It became apparent from these data that the precipitating antibody would produce an irritating complex with antigen in the region of antigen excess and none in antibody excess.

A similar study was made on a gamma globulin fraction from antiserum No. 3. The gamma globulin was first isolated by repeated precipitation with ⅓ saturated ammonium sulfate solution at pH 7.8. After dialysis a 4.0% solution was fractionated by starch block electrophoresis and eluates from 1.0 cm segments tested for precipitins and skin activity. The results were essentially the same as obtained previously for the whole antiserum. Again the slow moving gamma components which contained precipitin, but no skin reactive substance, were combined and aliquots mixed with varying amounts of antigen. The results are shown in Table I.

Some idea of the quantitative aspects of the activity of the soluble antigen-antibody complex was obtained from the following experiment. An antigen-antibody precipitate was formed by mixing a sample of the slow gamma obtained from antiserum No. 3 with an amount of BSA indicated in Table I at the equivalence point. The precipitate was washed 3 times with cold saline and then suspended in 2 ml of saline containing 128 μg N of BSA. The mixture was shaken for 18 hours at 4°C, the small amount of residue removed, and the solution tested for skin activity. Since the amount of antibody N in the original precipitate was approximately 78 μg N, the final solution contained less than 39 μg/ml. This solution gave a good positive skin reaction in dilutions of 1:16 and of 1:32. In order to obtain similar skin reactions with histamine phosphate, an amount of about 1.2 μg/ml was required. Assuming that the skin-reactive material from antigen-antibody mixtures was a soluble complex, then the limiting molecular weight would be of the order of 300,000 [$Ag_2Ab(4)$] and on a molecular basis would be more than 100 times as active as histamine.

B. *Activity of normal and immune serums without addition of antigen.* As was mentioned previously, serums without the addition of any antigen usually produced some local diffusion of dye into the skin of "blued" animals. In general, serums from normal rabbits gave a definite skin reaction in dilutions less than 1:10 while immune serums usually produced similar reactions in dilutions of 1:32 to 1:64. This effect of normal serum alone has been the subject of much investigation [*e.g.* Wilhelm *et al.*(5) who found an active component in alpha globulin]. However, in the present investigation the occurrence of the skin-reactive material in the gamma component of serum and the marked increase in activity following immunization suggests the possibility of some sort of antigen antibody mechanism.

The skin-irritating material of antiserum occurred in what is commonly considered fast gamma and slow beta (Fig. 1 and 2). It also occurred in the same general region of normal serum. This electrophoretic characteristic is essentially the same as found by Aladjem *et al.*(6) who obtained immediate-type skin reactions in normal human volunteers with fractions from immune rabbit serums. The skin reaction produced in the present study in blued guinea pigs was essentially the same as that produced by soluble antigen-antibody complexes. histamine or by the injection of antigen into the skin of a sensitized guinea pig. A positive reaction became quite definite in 2-3 minutes and reached a maximum in 30 minutes. The investigation of the nature of the skin-irritating material in immune and normal serum is not complete. However, preliminary studies indicate that it is a nondialyzable, heat-labile protein component that can be concentrated by centrifugation at 100,000 g as described for isolation of Rh agglutinating antibody by Campbell *et al.*(7).

Discussion. There seems to be no doubt that soluble antigen-antibody complexes found in excess antigen are toxic to guinea pigs and induce reactions which are similar to specific hypersensitivity. An explanation of the mechanism involved must await further study but the possible courses for investigation are obvious. In the case of soluble complexes the possibility exists that free antigen or antibody rapidly sensitizes tissue and the reaction observed is the result of a subsequent reaction with the complementary reagent (*e.g.* passive or reverse passive anaphylaxis). Another possibility is that the complex itself is toxic due to molecular changes brought about in either the antigen molecule or the antibody molecule as a result of combination. This leads to a study of many different antigen-antibody systems, including haptens, and to include soluble complexes formed in antibody excess. The third possibility is that the component of complement in antiserum is activated by the soluble antigen-antibody complex and becomes toxic; however, this was excluded by using decomplemented antiserum. Details of the experiment will be described later.

The skin-irritating fraction in normal and immune serum may be a circulating antigen-antibody complex. That of normal serum would not be known and would probably be a mixture of soluble complexes of a large number of antigens to which the animal is constantly exposed in his normal environment.

Summary. Immediate skin reactions were obtained in normal guinea pigs by intradermal injections of soluble antigen-antibody complexes. Immediate skin reactions were also obtained by injections of an electrophoretically separated component of serum from immunized or normal rabbits.

1. Trapani, I. L., Garvey, J. S., Campbell, Dan H., *Science,* in press.

2. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.,* 1949, v177, 751.

3. Ovary, Z., *Internat. Arch. Allergy and Immunol.,* 1952, v3, 112.

4. Singer, S. J., Campbell, Dan H., *J. Am. Chem. Soc.,* 1952, v74, 1794.

5. Wilhelm, D. L., Miles, A. A., MacKay, M. F., *Brit. J. Exp. Path.,* 1955, v36, 82.

6. Aladjem, F., MacLaren, W. R., Campbell, Dan H., *Science,* 1957, v125, 692.

7. Campbell, Dan H., Sturgeon, P., Vinograd, J. R., *ibid.,* 1955, v122, 1091.

Received January 13, 1958. P.S.E.B.M., 1958, v97.