THE BIOLOGICAL ACTIVITY OF SOLUBLE ANTIGEN-ANTIBODY COMPLEXES

II. Physical Properties of Soluble Complexes Having Skin-Irritating Activity*

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Previous work by Germuth and McKinnon (1), Trapani *et al.* (2), and ourselves (3) has established the fact that soluble antigen-antibody complexes formed in excess antigen can, (*a*) induce symptoms similar to anaphylaxis, (*b*) cause contraction of isolated smooth muscle from normal guinea pigs, and (*c*) increase the permeability of skin capillaries in a manner similar to that obtained in passive cutaneous anaphylaxis. These findings immediately raise many questions as to the fundamental mechanisms involved. For example, is the free antigen playing some role; is the toxicity dependent upon some change in the molecular structure of either antigen or antibody upon combination; is the complex itself toxic without any change in the molecular structure of the components; is the antigen-antibody ratio important; and, is complement involved? The work reported here involves a study of the possible role of free antigen and the nature of the complex. Some study was also made of untreated and decomplemented antiserums and, although there was no difference, this cannot rule out the possible participation of the test animal's (guinea pig's) own complement.

EXPERIMENTAL

General Procedures

Antigens.—The work to be reported here deals for the most part with bovine serum albumin (BSA) (Armour's crystallized). In order to determine antigen-antibody ratios in some of the experiments, samples of BSA were tagged with S^{35}-sulfanilic acid. The method of coupling and purification have been described by Garvey and Campbell (4). As a final stage in purification the S^{35}-BSA was developed on a starch block by electrophoresis and the material obtained from a 4 cm. area at the zone of maximum protein concentration was used for the immunochemical studies. The final preparation contained 1.8 sulfanilic groups per molecule

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of BSA and gave a quantitative precipitation curve which was essentially the same as that of unmodified BSA. Free boundary electrophoresis of the material in barbital buffer of pH 8.6 and ionic strength of 0.1 showed a single sharp peak which had a mobility of -6.3×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

Antiserums.—Four different pools (I, II, III, and IV) of rabbit anti-BSA serum were used. They varied in their content of precipitable antibody from 0.5 to 1.0 mg. of antibody nitrogen per ml. Immunization was the same in all instances; consisting of 10 mg. of BSA injected intravenously three times a week for 4 weeks. The animals were bled 7 days after the last injection. The four different pools merely represented four different groups of rabbits immunized at different times.

Skin Reactions.—All tests for skin irritability of complexes were made on guinea pigs by a modification of Ovary's method which we previously described (3). It consists essentially of first injecting a solution of Evans blue intravenously and then injecting 0.1 ml. samples of solutions of complexes into the skin of shaved back areas. Reactions are considered to be maximum between 15 and 30 minutes.

Starch Block Electrophoresis.—The starch block used in most of the present studies was 44 x 9 x 1.5 cm. The buffer used was barbital of pH 8.6, and ionic strength of 0.1 μ; the voltage was about 400 and the time was about 19 hours at an ambient temperature of 2°C.

RESULTS

A. Purification and Skin-Irritating Activity of Soluble BSA-Anti-BSA Complexes

Decomplementation.—These studies were made on a gamma globulin preparation isolated by salt precipitation and starch block electrophoresis from decomplemented serums I and II. Decomplementation of the antiserums was performed as follows:—

A washed precipitate of ovalbumin–rabbit antiovalbumin (140 μg. of antibody nitrogen per ml. of the anti BSA serum) was first suspended in the sample of anti BSA serum. The mixture was allowed to stand for 48 hours at 4°C. and stirred at intervals according to the method of Heidelberger *et al.* (5) and Maurer and Talmage (6). This procedure was repeated for each serum.

The treated serum showed no complement activity for sensitized sheep cells; it was also tested for coprecipitating material with an S II-rabbit anti-S II system. The protein content of a standardized S II precipitate was reduced from 0.154 mg. of nitrogen to a constant value of approximately 0.126 mg. of nitrogen after decomplementation or use of saline in place of untreated serum.

After decomplementation, a relatively pure preparation of anti BSA gamma globulin was obtained by repeated precipitation with ⅓ saturation of ammonium sulfate at pH 7.8. The final precipitate was redissolved in saline and dialyzed against 0.9 per cent NaCl containing a small amount of borate buffer of pH 8.4. After dialysis, 8.0 ml. samples containing about 4.0 per cent protein were further fractionated by starch block electrophoresis into slow and fast gamma components. This was done in order to separate the non-specific irritating substance (3) which occurs in the fast moving components of the gamma globulin of some serums from the precipitating antibody which occurs in the slow moving area. The block was cut into 1.0 cm. strips and the eluate from each was tested for protein concentration, pre-

cipitins, and skin-irritating activity. Eluates containing precipitating antibody and no skin activity were combined. Quantitative precipitin reactions were then carried out, using BSA for the preparation from serum I and S^{35}-BSA antigen for the preparation from serum II. After 48 hours at 4.0°C. the precipitates were centrifuged off and the precipitates and supernatants analyzed. The results are given in Table I.

TABLE I

Activity of Supernatants from Quantitative Precipitin Analyses

Antigen N added	Total N precipitated	Antibody N precipitated	Tests on supernate	
			Presence of Ag or Ab	Skin test (0.1 ml.)
μg.	μg.	μg.		
0.5 ml., slow γ-globulin fraction from decomplemented antiserum I, plus 0.5 ml. of BSA				
800	0	0	Excess Ag	−
200	0	0	" "	±
80	2.1	—	" "	+
40	6.1	—	" "	+++
20	13.3	—	" "	+++
10	35.4	*ca.* 25.4	" "	+++
5	35.4	30.4	Neither	−
2.5	17.6	15.1	Trace Ab	−
1.25	12.5	11.3	Excess Ab	−
0.63	5.8	5.2	" "	−
0	0	0	Antibody	−
0.5 ml., slow γ-globulin fraction from decomplemented antiserum II, plus 0.5 ml. of S^{35}-BSA				
185	0	0	Excess Ag	++
92.5	1.8	—	" "	+++
46.3	15.7	—	" "	+++
23.1	51.7	—	" "	+++
11.6	79.7	*ca.* 68.1	" "	+++
5.8	55.4	49.6	Excess Ab	−
2.9	34.5	31.6	" "	−
1.45	15.1	13.7	" "	−
0.73	5.75	5.0	" "	−
0	0	0	Antibody	−

Neither BSA (1.6 mg. N/ml.) or S^{35}-BSA (0.37 mg. N/ml.) produced skin reactions.

It will be seen that the active components occurred only in the supernatants from mixtures of moderate antigen excess and not in extreme antigen excess or antibody excess. These results were essentially the same as obtained with gamma globulin from untreated antiserum (3).

The activity of soluble complexes prepared by the addition of excess antigen to insoluble complexes was also tested.

Precipitates prepared at the equivalence points as shown in Table I, were washed with cold saline and then suspended in saline solution containing 2.6 and 2.3 times as much antigen as antibody in the precipitate, by weight. The suspensions were mildly agitated for 48 hours at

4.0°C. and then centrifuged to remove a trace of insoluble residue. The supernatant solutions (I and II) which contained a protein concentration of 0.676 mg. nitrogen per ml. and 0.816 mg. nitrogen per ml. respectively, were tested for skin activity.

The minimum amount of protein nitrogen to give a definite skin reaction was 2.1 and 1.3 μg. respectively. These findings are essentially in agreement with those previously described for untreated serums and suggest that complement is not important in the complex formed *in vitro*. However, this does not eliminate the possible participation of the complement of the test animal.

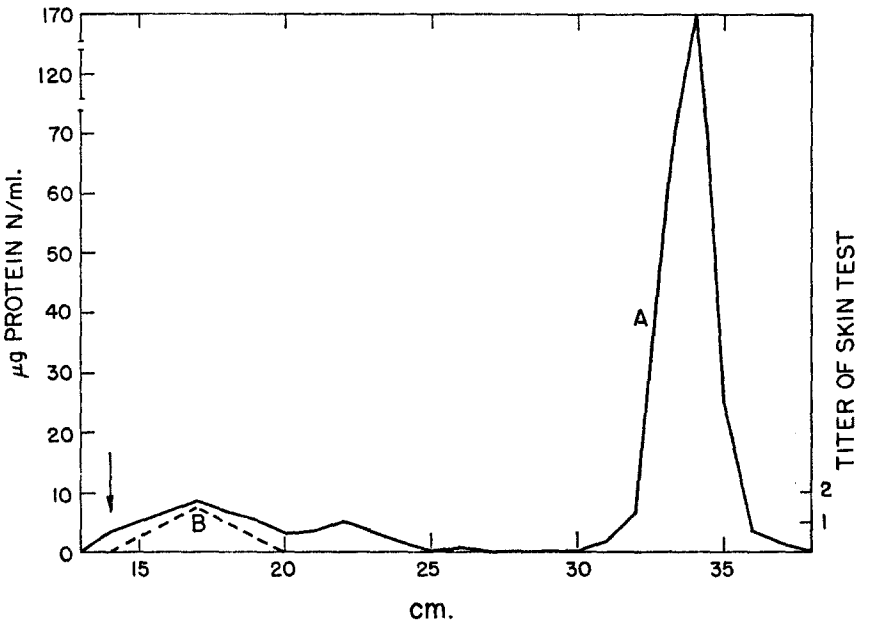

FIG. 1. Starch block fractionation of soluble complex (I). Electrophoresis was performed at pH 8.6, $\mu = 0.1$, 9 volts/cm., for 19 hours. Curve A represents protein concentration. Curve B represents titer of skin test, expressed by the maximum dilution, giving rise to definite reaction. Starting point is indicated by the arrow.

Activity of Soluble Complexes Purified by Starch Block Electrophoresis and Ultracentrifugation.—In order to further purify the skin-irritating complex, solutions I and II were subjected to starch block electrophoresis.

The sample used for I consisted of 8.0 ml. containing 0.676 mg. N per ml. The sample of II, concentrated by pervaporation, consisted of 8.0 ml. containing 1.63 mg. N per ml. The blocks were cut into 1.0 cm. segments and each eluted with saline. Analyses were then made on each eluate for protein concentration, skin activity, and, in the case of II, for S^{35}. The results are given in Figs. 1 and 2.

Since the slow gamma globulin fraction alone tended to migrate backward from the point of application, under the conditions used here, it became obvious

that in both experiments, the gamma globulin in the mixtures must have been combined with BSA. The resulting skin reactive component had a mobility between that of pure gamma, which has a low mobility, and BSA which has a high mobility. Eluates from this intermediate portion of the starch block, in the case of preparation I, gave skin reactions in amounts of 0.56 to 0.67 μg. N. Preparation II gave essentially the same result, except that protein concentrations were higher. Skin reactions with preparation II were obtained with 0.71

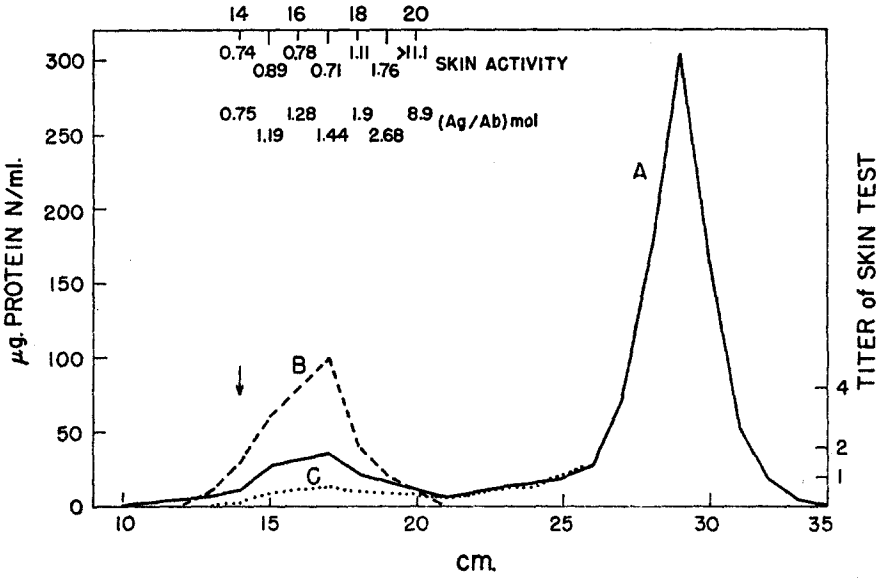

FIG. 2. Starch block fractionation of soluble complex (II). Electrophoresis was performed at pH 8.6, $\mu = 0.1$, 8 volts/cm., for 19 hours. Curve A represents protein concentration. Curve B represents titer of skin test. Curve C represents the concentration of S*BSA calculated from radioactivity. Starting point is indicated by the arrow. Skin activity of the active eluates, expressed by minimum skin doses (μg. protein nitrogen) and Ag/Ab molecular ratio in the eluates were illustrated on the upper part of the figure (see text).

to 1.76 μg. N by the eluates from 14 through 19. From S^{35}/N ratio, one can assume that only free antigen was present after about segment 21. The highest antigen-antibody ratio was slightly higher than theoretical, but this was probably the result of the very high excess of antigen in the system. It was noted in preparation II that segments 14 and 15 contained a small amount of an insoluble yellow material. This was probably an insoluble antigen-antibody precipitate formed by a re-equilibration reaction occurring during the electrophoresis (*cf.* Singer and Campbell (7, 8)).

On the assumption that the soluble complex should have a fairly high molecular weight, a sample of preparation II (combined eluates from segments 14 through 19) was centrifuged for 4 hours at approximately 100,000 *g* in a Spinco preparatory centrifuge (9). Using centri-

fuge tubes which held approximately 12 ml., the centrifuged solutions were divided into four fractions namely, II_{-1} the 4 ml., II_{-2} the next 4 ml., II_{-3} the lower 4 ml., and II_{-4} the pellet plus a small amount of solution. The pellet was dissolved in 12 ml. of saline and recentrifuged. The centrifuged solution was again fractionated in the same manner to give II_{4-1}, II_{4-2}, II_{4-3}, and II_{4-4}. The latter consisted essentially of the gelatinous pellet which was then dissolved in 4 ml. of 0.9 per cent saline. The controls consisted of (*a*) a sample of the same solution of soluble complex II as that used for starch block electrophoresis and (*b*) a solution of S^{35}-BSA. The results are given in Table II.

Skin-reactive material was partially concentrated in the pellet from both the starch block eluate and the preparation II containing excess antigen. The "heavy" component from both systems contained a trace of insoluble yellow

TABLE II

Skin Activity of Soluble Complex Obtained by Ultracentrifuge

Fraction	Eluate			Solution II			S^{35}-BSA	
	Total protein	BSA (S^{35})	Skin* activity	Total protein	BSA (S^{35})	Skin activity	S^{35}-BSA	Skin activity
	μg. N/ml.	*μg. N/ml.*	*μg. N.*	*μg. N/ml.*	*μg. N/ml.*	*μg. N*	*μg. N/ml.*	*μg. N*
II_{-1}	*Ca.* 4	3.1	—‡	144	125	7.2	96	—‡
II_{-2}	" 4	2.7	—	164	154	4.1	196	—
II_{-3}	" 4	3.2	—	228	179	2.9	132	—
II_{4-1}	—	0.8	—	68	43	1.7	12	—
II_{4-2}	—	1.2	—	76	41	1.9	8	—
II_{4-3}	4	1.6	±	92	50	1.2	12	—
II_{4-4}	56	12.0	0.50§	296	58	0.75§	4	—

* Skin activity was expressed by the minimum amount of protein N required to cause a definite skin reaction.

‡ Negative; required more than the concentration of nitrogen in the sample tested.

§ After centrifuging off the precipitate at 3000 R.P.M. for 30 minutes, the supernatant was tested for skin activity. $(Ag/Ab)_{weight}$ of the supernate from Fr. II_{4-} of solution II was 0.67.

precipitate which would suggest re-equilibration in a system poor in free antigen. The antigen-antibody ratio on the basis of nitrogen was essentially the same for both II_{4-4} fractions. This ratio is also close to the lowest ratio in the eluate from starch block electrophoresis. However, the skin activity of II_{4-4} fraction obtained from the starch block eluate was slightly higher.

Comparison of the Amount of Antigen and Antibody Required for Passive Cutaneous Anaphylaxis with That of Active Preparations of Soluble Complexes.— Although it would appear from the previous experiments that the soluble antigen-antibody complex itself was toxic, the possible role of small amounts of dissociated antigen or antibody must be considered. An investigation was therefore made to determine the amounts of either antigen or antibody which were required for reverse or direct passive sensitization of the skin.

One-tenth ml. aliquots of various concentrations of either the slow gamma from serum II or a solution of BSA were injected into the skin of "blued" guinea pigs in 0.1 ml. volumes. This was immediately followed by an injection of the complementary agent into the same area. The results are given in Table III.

The limiting amount of antigen which was required for reverse sensitization was about 0.2 μg. N, this in turn required 0.75 μg. N of antibody to give a reaction. These values may seem high, but are due to the relatively short time interval between the primary and secondary injections. The amounts required are of the same order as those for the total soluble complexes. It would seem therefore, that the small amounts of either free antigen or antibody that might be expected to be present in a preparation of soluble complex as a result of chemical equilibrium, play little or no part in the skin reactions. From the results described in a following section (see Fig. 3 and Table V) the amount of free antigen (or antibody) would be of the order of 10 per cent or less of the soluble complex. Since most complexes studied so far are active in the range of 0.5 to 0.8 μg. N, the value of free antigen would be around 0.07 μg. N, which is less than the limiting amount required for reverse sensitization. This experiment was repeated using antibody from serum III with essentially the same result.

The Effect of the Local Injection of Antigen on the Skin Activity of Soluble Complex.—As will be noted in Table I, as well as from work reported previously by others as well as ourselves, the complexes formed in extreme antigen excess have little or no activity. Although we believe that inactivity was the result of some difference in the complex itself, the possibility arose that the free antigen might be acting in some way as an inhibitor. To test this possibility, "blued" guinea pigs were first injected intradermally with 0.05 ml. of saline containing 40 or 80 μg. N BSA. This was immediately followed by a 0.1 ml. of saline containing amounts of purified complex III (see below) which varied from 0.39 to 6.3 μg. N. In no instance was there any apparent inhibition of the skin activity of the complex.

B. Physical Properties of the Active Soluble Complexes

These studies were carried out with soluble complexes prepared from serums III and IV as described by Singer and Campbell (7).

The gamma globulin fractions were first removed by precipitation with ⅓ saturated ammonium sulfate at pH 7.8 and purified by repeated precipitation and subsequent dialysis until free of ammonium sulfate. The antibody was then precipitated at the equivalence point with antigen (III with S^{35}-BSA and IV with BSA). After 48 hours at 4°C., the precipitates were removed, washed, and dissolved in a saline solution containing an excess of antigen of approximately two times the weight of the antibody present. The two systems were then gently mixed at 4°C. for 72 hours with constant mixing and then centrifuged to remove a trace of residue which remained. The soluble complexes were then freed of most of the excess antigen by precipitation with ½ saturated ammonium sulfate at pH 7.8, then redissolved and dialyzed until free of sulfate.

Dependence of the Skin-Irritating Activity on the Composition of the Complex.— The effect of change in composition of a complex by the addition of antigen on the skin activity was shown by the following experiment.

A sample of preparation III was divided into four aliquots. One volume of saline was added to one, and to the others, one volume of saline containing 4.05, 8.10, and 16.20 mg. N of BSA, respectively. The four samples were labeled III, III_{-1}, III_{-2}, and III_{-3}. The antigen/antibody ratio of III was determined by radioactivity and total protein analysis and the others from

TABLE III

Amounts of Antigen and Antibody for an Immediate Sensitization

Anti-BSA		Micrograms of BSA N						
		1.6	0.8	0.4	0.2	0.1	0.05	025
*μg. N**								
6.0	Passive‡	+++	+++	++	++	+	±	±
	Reversed§	+++	++	++	±	−	−	−
3.0	Passive	+++	+++	++	+	−	−	−
	Reversed	+++	++	+	±	−	−	−
1.5	Passive	+++	+++	++	+	−	−	−
	Reversed	++	++	+	+	−	−	−
0.75	Passive	+++	+++	++	+	−	−	−
	Reversed	++	++	++	+	−	−	−
0.5	Passive	+++	+++	++	+	−	−	−
	Reversed	+	+	−	−	−	−	−

* Actual amount of Ab N in 0.1 ml. of slow gamma globulin.

‡ Antibody injection followed by antigen.

§ Antigen injection followed by antibody.

antigen added and total protein. Skin tests were made with samples taken 15 minutes after mixing and again at 10 days to allow for equilibrium to be reached. The results as shown by the minimum skin reactive dose at ten days are given in Table IV.

The effect of allowing the systems to reach equilibrium was to increase slightly the amount of complex required. One can assume that under these conditions, all the antibody must be in a complex of some form and that as the composition of the complexes change to a higher antigen/antibody ratio, the skin activity decreases.

A similar experiment was carried out with preparation IV.

Three different antigen/antibody ratios were studied; IV to which only one volume of saline was added, and IV_{-1} to which one volume of saline was added but contained 20.15 mg. N BSA/ml. These were allowed to stand for 14 days at 4°C. Preparation IV_{-2} was obtained from IV_{-1} by precipitation with ½ saturated ammonium sulfate at pH 7.8, then dissolved

in barbital buffer pH 8.6, $\mu = 0.1$, and dialyzed free of ammonium sulfate for 2 days. The minimum skin reactive dose of these preparations are also given in Table IV.

Here again, the amount of nitrogen required for the minimum skin reacting dose of complex (based on total Ab) is higher in antigen excess (IV_{-1}). When excess antigen was removed from the IV_{-1} system (IV_{-2}) the activity again increased due to re-equilibration.

An idea of the electrophoretic composition of these various complexes is given in Tables V to VII and Figs. 3 and 4. The free-boundary electrophoretic studies were all made with a Perkin-Elmer electrophoresis apparatus (model 38) using barbital buffer (pH 8.6, $\mu = 0.1$) unless otherwise stated. Some repre-

TABLE IV

*Analysis of the Components in the Minimum Skin Doses of Soluble Antigen-Antibody Complexes**

Solution No.	Total protein	(Total Ag/total Ab)$_{weight}$	Total Ag	Total Ab	Free‡ Ag	Total† Complex	Complex 1‡	Complex 2‡
	$\mu g.N$		$\mu g.N$	$\mu g.N$	$\mu g.N$	$\mu g.N$	$\mu g.N$	$\mu g.N$
III	0.78	0.64	0.30	0.48	0.07	0.71	0.04	0.66
III_{-1}	3.00	3.28	2.30	0.70	1.88	1.12	0.38	0.74
III_{-2}	6.60	5.94	5.65	0.95	4.98	1.62	0.95	0.67
III_{-3}	23.40	11.20	21.50	1.92	19.90	3.52	2.79	0.73
IV	0.85	0.60	0.32	0.53	0.10	0.75	0.05	0.70
IV_{-1}	54.00	12.90	50.10	3.90	46.80	7.24	6.30	0.94
IV_{-2}	1.48	1.24	0.82	0.66	0.44	1.04	0.09	0.95

* All values are based on a dose of 0.1 ml.

‡ These values were calculated from the results of electrophoretic analysis (*cf.* Table V), assuming that the relative concentration of each component does not change by dilution.

sentative patterns are given in Fig. 3. These were made of the preparations III, III_{-1}, III_{-2}, and III_{-3}. The fast moving complex is arbitrarily designated as 1 and the slower as 2 in the present discussion, which would correspond to the *a* and *b* + *h* complexes described by Singer and Campbell (7). Because of the re-equilibration of antigen-antibody components, the curve between free antigen and complex is elevated from the baseline in the descending diagram. Mobility values and concentrations of components have been listed in Table V. It was apparent that the mobility of complex 1 was approximately constant under the conditions studied while that of 2 changed as the antigen-antibody concentration in the total system changed. It was also apparent that the minimum skin reactive dose of complex 2 was essentially constant although the amount of the component itself changed significantly (Table IV). This would suggest that the significant component as far as skin activity was concerned was complex 2. It should be noted that the soluble complexes split into two electrophoretic components. In preparation III the peak representing the complex, was distorted toward the higher mobility side, but no real double maximum occurred.

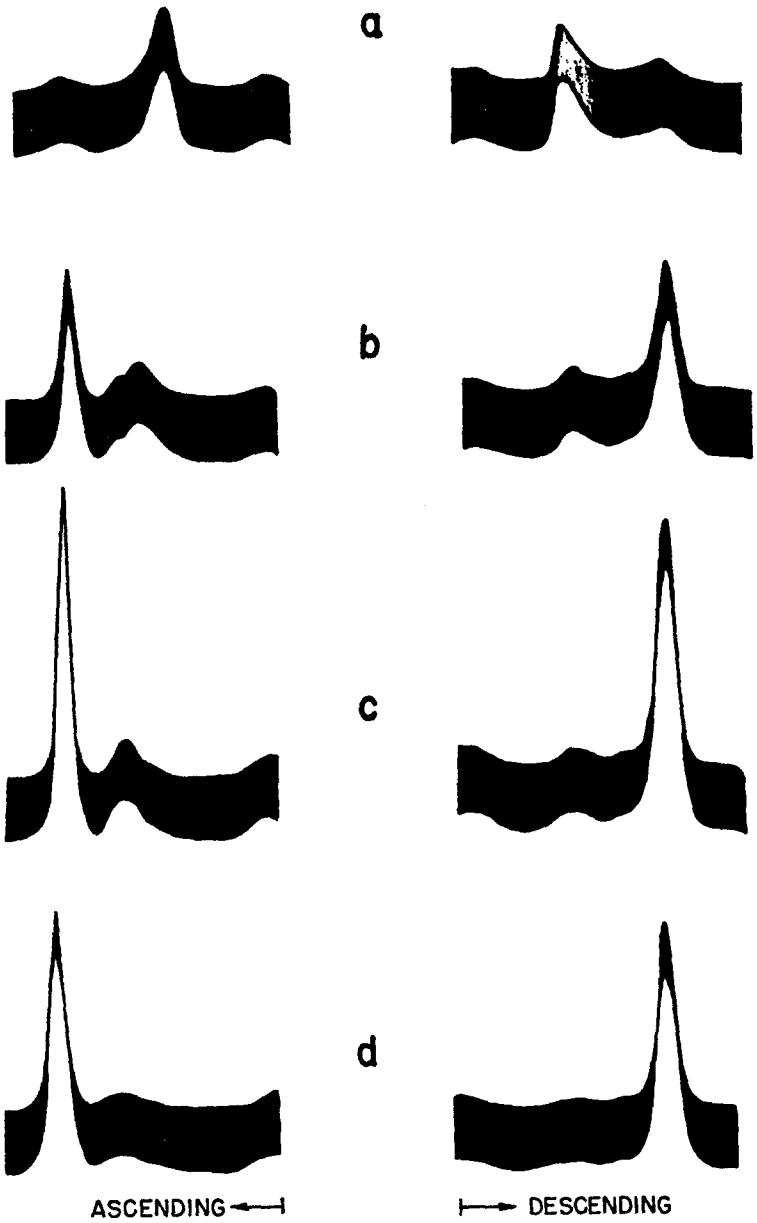

FIG. 3. Electrophoresis patterns of soluble complexes. In barbital buffer pH 8.6, $\mu = 0.1$, 7200 seconds at 0.006 amp. (*a*) solution III, (*b*) solution III_{-1}, (*c*) solution III_{-2}, (*d*) solution III_{-3}. Starting points are indicated by the arrows.

In preparation IV_{-2}, complex 2 was predominant, although it was obtained from IV_{-1} in which it was the minor component (*cf.* Table V). This was probably the result of re-equilibration established during isolation and dialysis. The relative amount as well as the mobility of complex 2 in this preparation is different from that obtained for III, III_{-1}, and IV. In these latter solutions the mobility of complex 2 is -3.5 to -3.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, whereas in IV_{-2} it is $-4.0 \times$ cm.^{2} volt^{-1} sec.$^{-1}$ in spite of the relatively low free antigen. This suggests that the complexes in the solution of IV_{-2} had not yet reached equilibrium. As shown in columns 7 and 9 of Table IV, the total amount of complex in the minimum skin dose of IV_{-2} was much less than that of IV_{-1}

TABLE V

Electrophoresis of Soluble Antigen Antibody Complexes

Solution No.	Mobility*						Relative area‡		
	Ascending			Descending					
	Antigen	Complex 1	Complex 2	Antigen	Complex 1	Complex 2	Antigen	Complex 1	Complex 2
							per cent	*per cent*	*per cent*
III	7.2	—	3.5	6.3	—	2.5	9.2	(4.9)	85.9
III_{-1}	7.3	4.8	3.8	6.2	—	3.0	63.5	12.7	24.6
III_{-2}	7.2	4.9	4.0	6.3	4.5	3.0	75.5	14.4	10.2
III_{-3}	7.2	4.9	3.9	6.3	4.5	3.1	85.0	11.9	3.1
IV	7.2	—	3.5	6.3	—	—§	12.1	(5.8)	82.1
IV_{-1}	7.3	4.8	4.0	6.3	4.6	2.9	86.6	11.7	1.74
IV_{-2}	7.2	4.9	4.0	6.2	—	—§	29.5	6.3	64.2

( ) Estimated from the asymmetry of the leading edge of the complex peak.

* In units of -10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

§ Peak was not clear because of the appearance of precipitate.

‡ Calculated from ascending diagrams.

although the same amount of complex 2 was contained in both. Again it became apparent that the activity was dependent upon complex 2 rather than 1.

In order to determine total antigen/antibody ratio in solution IV, IV_{-1}, and IV_{-2}, they were dialyzed against glycine-HCl buffer (pH 2.35, $\mu = 0.1$) for 2 days and electrophoresis was performed at the same pH. The electrophoretic diagrams are shown in Fig. 4. From descending diagrams, relative concentration of BSA and antibody were calculated. In order to eliminate uncertainties due to electrophoretic anomalies, the relative areas under the peaks were calibrated by experiments with accurately prepared mixtures of BSA and normal rabbit gamma globulin (Singer and Campbell (10)). A normal rabbit slow gamma globulin preparation, obtained by starch block electrophoresis, gave a single electrophoretic boundary at pH 2.35. BSA and the gamma globulin fraction were mixed in varying ratio. The mixtures were dialyzed against glycine-HCl buffer and analyzed by free boundary electrophoresis at a protein concentration of 1.9 mg. N/ml. The apparent relative area under each peak in the descending patterns was determined. Comparisons of the apparent area of BSA and its actual per cent in

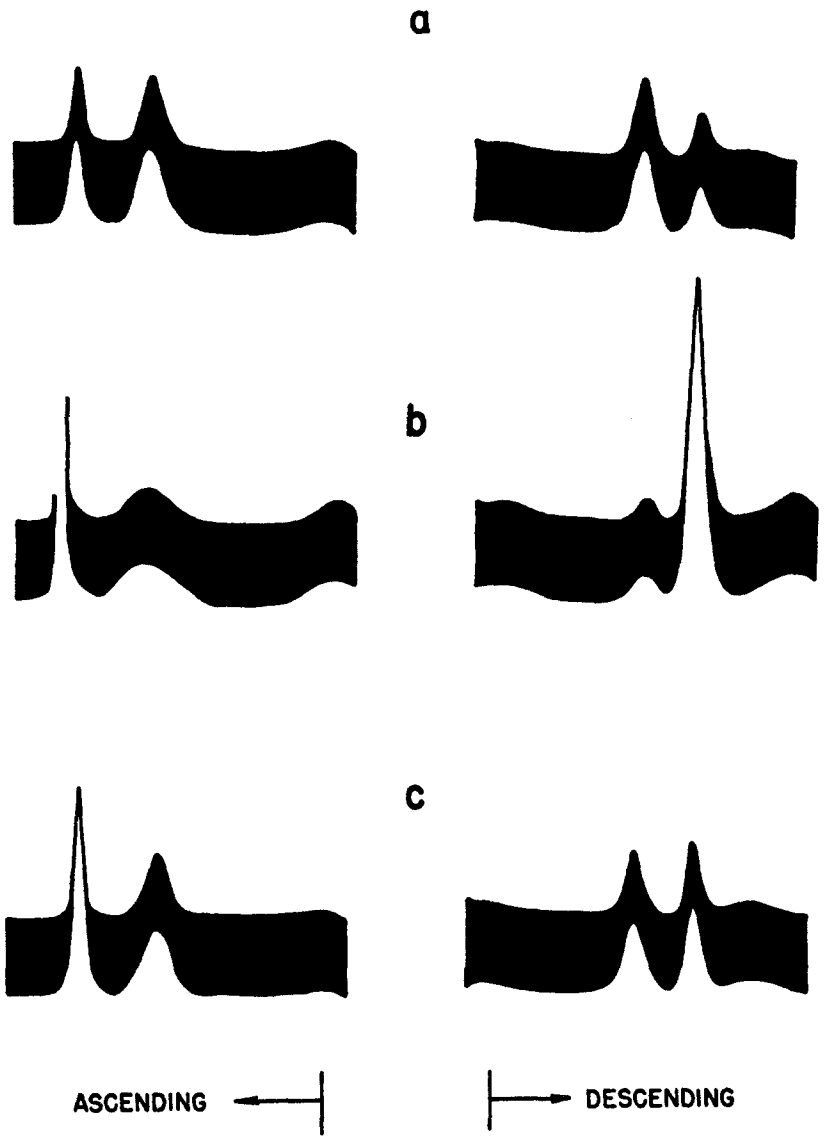

FIG. 4. Electrophoresis diagrams in glycine-HCl buffer, pH 2.35, μ = 0.1, after 7200 seconds at 0.008 amp. (*a*) solution IV, (*b*) solution IV_{-1}, (*c*) solution IV_{-2}. Starting points are indicated by the arrows.

each solution are listed in Table VI. The percentage of total BSA in solution IV, IV_{-1}, and IV_{-2} (Table VI, column 4) was then obtained with the aid of the calibration data.

These results indicate that the skin activity of the soluble antigen-antibody complexes depends on the concentration of complex 2 ($\mu = -3.5$ to 4.0×10^{-5}

cm.^{2} volt^{-1} sec.$^{-1}$), and that complex 1 ($\mu = -4.8 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$) has no activity.

DISCUSSION

The data presented in this report clearly indicate that the BSA–anti-BSA soluble complexes, formed in antigen excess, have skin irritability and that the toxicity depends on their composition. The electrophoretic studies on the soluble complex preparations (III and IV) indicate that the slower moving complex (complex 2) rather than the rapidly moving complex (complex 1) is involved in the skin reaction. It has been shown by the work of Singer and Campbell (7) that the electrophoretic mobility of the soluble complexes depends on their composition. The mobility of complex 1 is the same as the

TABLE VI

Electrophoretic Analysis of BSA Gamma Globulin Mixtures in Glycine-HCl

No.	Composition	BSA	
		Apparent	Analytical
		per cent	*per cent*
1	BSA + normal γ-globulin	26.0	35.4
2	" + " "	32.8	38.4
3	" + " "	50.8	57.7
4	" + " "	62.5	68.0
5	" + " "	89.0	92.4
IV	BSA–anti-BSA	29.4	(37.5)
IV_{-1}	"	89.4	(92.8)
IV_{-2}	"	48.7	(55.3)

( ) Calculated from calibration curve.

mobility of the Ag_2Ab complex reported by them. Also, the theoretical concentrations of Ag_2Ab complex in preparation III, III_{-1}, III_{-2}, and III_{-3} and IV, IV_{-1}, calculated following Singer and Campbell (11), were in good agreement with the concentration of complex 1. These results indicate that complex 1 is an Ag_2Ab complex. Complex 2 would be due to a mixture of species poorer in antigen than Ag_2Ab. Following Singer and Campbell (7), $(\overline{Ag/Ab})_{NB}$, the average number of antigen molecules bound per antibody in all of the complexes, was calculated. The values of $(\overline{Ag/Ab})_{NB}$, listed in column 4 of Table VII, increase regularly as the antigen excess is increased. Assuming that complex 1 is Ag_2Ab, the antigen and antibody in complex 1 were subtracted from the total antigen and antibody respectively, which are bound in all of the complexes. This permits the calculation of values of $(\overline{Ag/Ab})'_{NB}$, the average number of antigen bound per antibody in complex 2 (column 5 of Table VII). This value increased with increased antigen concentration and paralleled the increase of electrophoretic mobility of complex 2 (Table V). In the case of preparation

III_{-3} and IV_{-1}, which are in extreme antigen excess, $(\overline{Ag/Ab})'_{NB}$ was 1.54 and 1.66 respectively, indicating that complex 2 of these solutions is mostly Ag_3Ab_2. As the skin irritating activity of the soluble complex preparation depends on the concentration of complex 2 and not upon the concentration of complex 1, it is obvious that the Ag_2Ab complex does not have the activity, while the Ag_3Ab_2 complex and more complicated complexes do. Inactivity of Ag_2Ab complex was assumed by Tokuda and Weiser recently (12). In their experiment however, the addition of excess antigen to the soluble complex preparation did not diminish its activity to elicit shock in mice. This finding is contradictory to their assumption and our present findings. Such a discrepancy might be due to their method of preparing soluble complex preparations. In their

TABLE VII

Composition of Soluble Antigen-Antibody Complexes

Solution No.	$(\text{Total Ag/total Ab})_{mol}$	Free antigen	$\overline{(Ag/Ab)}^*_{NB}$	$\overline{(Ag/Ab)}'_{NB}$‡
		per cent		
III	1.46	9.2	1.10	1.06
II_{-1}	7.50	63.5	1.30	1.12
II_{-2}	13.4	75.5	1.64	1.26
III_{-3}	25.6	85.0	1.98	1.54
IV	1.37	12.1	0.93	0.88
IV_{-1}	29.5	86.6	1.87	1.66
IV_{-2}	2.84	29.5	1.32	1.25

* Average molecular ratio in all complexes.

‡ Average molecular ratio in complex 2.

preparations, the soluble complexes might not be in equilibrium because of too short a period of incubation.

An explanation of the mechanism of the toxicity of the soluble complexes must await further study. As described in the previous report (3), one of the possibilities is that free antigen or antibody rapidly sensitizes tissue and the reaction observed is the result of passive or reversed passive anaphylaxis. However, the result of the present experiment excluded this possibility. From the present data it becomes obvious that (*a*) purified soluble complexes have the activity, (*b*) free antigen contained in the purified soluble complex preparation is not sufficient to sensitize guinea pig skin, and (*c*) the threshold amount of antigen or antibody required to elicit a skin reaction when challengd as quickly as possible by the complementary reagent, is in the same order of magnitude as the amount contained in the minimum skin dose of soluble complexes (Table III). If the mechanism of the skin reaction with the soluble complex involves passive or reversed passive anaphylaxis, all the antigen and antibody in the soluble complexes would have to dissociate and recombine in

the tissues within 2 to 4 minutes, because the skin reaction becomes quite apparent in a few minutes after the intradermal injection of soluble complexes (3). Even when histamine is injected intradermally, about the same period is required to show the definite reaction. Taking the low dissociation constant and slow rate of re-equilibration of the antigen-antibody reaction into consideration (13), passive or reversed passive anaphylaxis could not be the correct mechanisms. Aggregation of the soluble complexes in the tissues, to damage the cells mechanically, could not be the mechanism of their toxicity since injection of insoluble complexes produces no reaction. One might think that after the soluble complexes are injected into the tissues, entirely new equilibria may exist in the tissues by the rapid disappearance of free antigen from the injected site and the complexes produced under these circumstances might give the skin reaction. However, the prior treatment of the site with a large amount of antigen showed no inhibition on the toxicity of the complex. If the skin reaction should be due to the re-equilibration of antigen-antibody in the tissues, free antigen in the tissue would influence the reaction. The dependence of the skin activity on the composition of the soluble complexes also suggests that the re-equilibration is not the case.

Another problem to consider in the formulation of the mechanism of the skin reaction is the role of complement. The present experiments show that the purified soluble complexes prepared from decomplemented antiserums are toxic and the minimum skin doses of the purified complexes (0.75 to 0.5 μg. N in Table II) are about the same as that prepared from non-decomplemented serums (preparation III and IV in Table V). These findings indicate that complement is not necessary for the formation of active complexes *in vitro*, although the role of tissue complement in the skin reaction cannot be excluded. As suggested by Osler *et al.* (14), tissue complement may play some role in the production of the PCA[1] reaction in rat skin. Our recent studies which will be published later, on the complement fixability of the soluble antigen-antibody complexes indicate that the Ag_2Ab complex of the BSA system does not fix complement and that the skin activity of the supernatants from the precipitin reaction roughly parallel with their complement fixability. These results suggest the role of tissue complement or a complement-like substance in the skin reaction. This may be more apparent than real since some antigen-antibody systems which fix complement do not give a skin reaction (*e.g.* SII–anti-SII system).

A reasonable possibility for the mechanism of the skin reaction is that the soluble complex becomes toxic owing 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. The data obtained in the recent studies, which will be published later, show that the soluble complexes of the artificial dye hapten (R_3')-antiarsanil azoprotein

[1] Passive cutaneous anaphylaxis.

rabbit antibody and of SIII–anti-SIII rabbit antibody give the skin reaction. On the other hand, the soluble complexes of diphtheria toxoid-horse antitoxin do not have the activity, whereas, diphtheria toxoid-rabbit antitoxin complex has high activity. These findings suggest the importance of the property of antibody rather than antigen in the formation of the active complexes. However, sodium arsanilate–antiarsanil–azoglobulin complex does not have activity even though rabbit antibody is used.

Since the Ag_2Ab complex of the BSA system is also inactive, it seems that the participation of two or more antibody molecules is required for the formation of active complexes. Studies on the role of antibody in immune hemolysis by Weinrach and Talmage (15) suggest the requirement of the combination of two or four antibody molecules with adjoining sites on the red cell to give the complex EA_2 or EA_4 for hemolysis. This hypothesis reminds us of the idea that the combination of two or more antibody molecules to one antigen molecule, on its adjoining sites, might induce the structural change of antibody and/or antigen and the complex might become toxic. We therefore visualize that the molecular configuration of antibody in a complex, with a ratio such as Ag_3Ab_2 or lower, might be changed in some systems to form a toxic configuration. This change may be physical, such as partial unfolding of certain portions of the polypeptide chains to produce new steric groups, or a change in the chemical nature of the antibody brought about by masking or freeing certain groups (*e.g.* $-COOH$ or $-NH_2$) to give the molecule a different character. The final structure may be such as to give some of the complex properties of a histamine-like substance or a histamine liberator. In any event, it is of interest that such soluble complexes prepared *in vitro*, give allergic-like responses and suggests that soluble complexes formed by antigen and reagin in human beings may function in a similar manner.

SUMMARY

Soluble BSA–anti-BSA complexes, formed in antigen excess, give immediate skin reactions in normal guinea pigs. The mechanism of the reaction is not that of passive or reversed passive anaphylaxis. The complex itself is toxic.

Skin activity of the complex depends on its composition. It has become obvious that the complex composed of two antigen molecules and one antibody molecule, (Ag_2Ab), does not have the activity, whereas, Ag_3Ab_2 and more complicated complexes do.

The role of complement as well as speculation on the structural changes of antibody-antigen complexes is presented.

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