[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, INDIANA UNIVERSITY]

The Dissociation of Antigen–Antibody Precipitates[1]

BY FELIX HAUROWITZ, RAYMOND SOWINSKI[2] AND HSIEH FU CHENG

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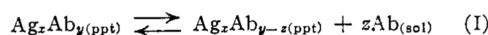
In a number of antigen–antibody systems, in which azoproteins were used as antigens, the insoluble antigen–antibody complex was suspended in 0.9% saline solution and allowed to equilibrate. Using isotopically labeled radioactive antigen (Ag) or Antibody (Ab) it was found that the saline extract of the precipitates contained antibody protein, but no significant amounts of antigen. More antibody was extracted from the precipitates at 25° than at 3°. It is assumed that the following reaction takes place: $Ag_xAb_{y(ppt)} \rightleftarrows Ag_xAb_{y-z(ppt)} + zAb_{(sol)}$. The concentration of Ab in the saline extracts was determined and equilibrium constants for different ratios of y/x were computed. ΔF for the combination of Ab with various antigen–antibody complexes is approximately −8 to −10 kcal. per mole of antibody. The entropy of combination is positive; it is lower in the equivalence zone than in an excess of Ag or Ab.

The precipitation of antigen (Ag) with rabbit antibody (Ab) leads to the formation of precipitates of the general formula Ag_xAb_y. Numerous attempts have been made to correlate the ratio y/x and the amount of precipitate with the concentrations of Ag and Ab in the supernatant solution. As a result, various theoretical or empirical equations have been proposed by Heidelberger and Kendall,[3] Pauling,[4] Goldberg,[5] Teorell[6] and other investigators. It is difficult to prove the validity of any of these equations over the whole range of antigen–antibody ratios for the following reasons: (1) in the region of antigen excess the precipitate is soluble; the solution contains free antigen and a variety of soluble antigen–antibody complexes of different composition; (2) in antibody excess the solubility of the precipitate is extremely low and can hardly be measured; (3) some of the antibody molecules are not precipitated by antigen although they are co-precipitated in the presence of true, precipitating, antibodies. The terms "reagin," "low-grade antibody" or "univalent antibody" have been used for these non-precipitating antibodies.

The antigen–antibody equilibria in the region of antigen excess have been investigated by Singer and Campbell.[7] In the present paper the equilibrium between antigen and antibody in the region of antibody excess is examined. Our investigation is based on the observation[8] that antigen–antibody precipitates on repeated washing with 0.9% saline solution dissociate into free antibody and an insoluble antigen–antibody complex according to reaction I



$$Ag_xAb_{y(ppt)} \rightleftarrows Ag_xAb_{y-z(ppt)} + zAb_{(sol)} \quad (I)$$

where z is smaller than y.

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(3) M. Heidelberger and F. E. Kendall, *J. Exp. Med.*, **62**, 467 (1935).

(4) L. Pauling, D. H. Campbell and D. Pressman, *Physiol. Rev.*, **23**, 203 (1943).

(5) R. J. Goldberg, THIS JOURNAL, **74**, 5715 (1952).

(6) T. Teorell, *J. Hygiene*, **44**, 227, 237 (1946).

(7) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952); **77**, 3499, 4851 (1955).

(8) F. Haurowitz, C. F. Crampton and R. Sowinski, *Federation Proc.*, **10**, 560 (1951).

The antigens used in our experiments were azoproteins containing the determinant group $-N=N-C_6H_4X$, where X was the *o*-carboxylic, *p*-arsonic or *p*-sulfonic acid group. Since the concentration of antigen and antibody in washings and in saline extracts of the precipitates was very low, we labeled antigen or antibody in some of the experiments with radioactive isotopes and determined their concentration by measuring the radioactivity.

Experimental

Antigens.—The preparation of beef serum pseudoglobulin, its coupling with 0.70 mmole arsanilic, anthranilic or sulfanilic acid per gram of protein, and the purification of the azoproteins was described earlier.[9] Similarly, beef serum γ-globulin or crystalline ovalbumin were coupled with the diazotized aromatic amino acids. The three azoglobulins obtained are designated as AsBG, AnBG and SuBG, respectively, the analogous ovalbumin derivatives as AsOv and SuOv.

Immune Sera and Antibodies.—Rabbits were immunized and killed as described previously.[9] Sera of similar, high titer were pooled, kept for one hour at 55° to inactivate complement, chilled and centrifuged in the cold. After removal of lipid particles by filtration in the refrigerator, the sera were stored at −12°. In some of the experiments the globulins were precipitated from the serum by 22% sodium sulfate at 37°, dialyzed and dissolved in 0.16 *M* sodium phosphate buffer solution *p*H 7.0 for labeling with iodine. In other experiments purified antibodies were prepared by adding the optimum amount of antigen to the γ-globulin solutions, washing the precipitates four times with saline solution and dissociating them by treatment with sodium chloride and hydrochloric acid.[10] The purified final solutions contained approximately 1 mg. antibody per ml.

Labeling with I^{131}.—A method similar to that of Pressman, *et al.*,[11] was used to label AnBG (Table I) and AsBG (Table II) with traces of I^{131}. The γ-globulins from 77 ml. of the pooled anti-AnBG sera 107/110 and 121/3 were precipitated by 22% sodium sulfate at 37°, dissolved in 70 ml. of phosphate buffer and treated with 0.15 mmole (2 millicurie) of iodine. In a parallel experiment, non-radioactive I^{127} was used in order to eliminate secondary effects of iodination. From both globulin solutions purified antibody was prepared as described above. The γ-globulins from 38 ml. of anti-AsBG serum 114/6 were treated with 0.2 millimole (5 mc.) of iodine and used without further purification (Table III).

I^{131} activities were measured by placing 1 ml. of the suitably diluted solution on an aluminum planchet, adding a drop of very dilute soap solution, drying and counting under a window counter. Precipitates were dissolved by heating in 1% NaOH solution, and evaporated on copper planchets. Radiation measurements on antigen, antibody, precipitates

(9) F. Haurowitz and P. Schwerin, *J. Immunol.*, **47**, 111 (1943).

(10) F. Haurowitz, S. Tekman, M. Bilen and P. Schwerin, *Biochem. J.*, **41**, 304 (1947).

(11) D. Pressman and L. A. Sternberger, THIS JOURNAL, **72**, 2226 (1950).

and washings were all made on the same day so that correction for background, scattering and absorption effects were minimized. The activities of the four preparations labeled by I^{131} were (in counts/min./mg.): AnBG 4954, purified anti-AnBG 3450; AsBG 286,000 (Table II); anti-AsBG 11,310 (Table III).

Dissociation of Radioactive Precipitates.—In the experiment shown in Table I each of the 12 tubes contained 2 ml. of purified antibody solution and 0.1 mg. of the antigen in 8 ml. of 0.9% saline solution. One set of 6 tubes contained radioactive antigen, Ag*, and non-radioactive antibody, Ab; the other six tubes contained radioactive antibody, Ab*, and non-radioactive antigen, Ag. The non-radioactive Ag and Ab contained I^{127} instead of I^{131}. In experiment no. 1 the antigen–antibody mixtures were kept for 20 hours at 3.5°, equilibration between precipitates and supernatant solutions being facilitated by occasional stirring. Tubes a and b were stored 4 more hours at 3.5°, tubes c and d warmed up during 30 minutes and kept 3.5 hours at 23° in order to allow equilibration at this temperature. Centrifuging of the equilibrated suspensions at 3.5 and 23° gave four supernatants and four precipitates. In experiment no. 2 all four tubes were kept 24 hours at 3.5°, then centrifuged and the supernatant solution discarded. The precipitates were suspended in 10 ml. of 0.9% saline solution, kept for 20 hours at 3.5°, then treated for four hours in the same manner as in experiment no. 1. In experiment no. 3 the 24-hour extraction with saline solution was repeated; both supernatant solutions were discarded; the extracted precipitates were then suspended in 10 ml. of saline solution and treated as in exp. no. 1. The 12 precipitates were washed with 50% methanol, pure methanol, acetone and ether[9] and dried. Ag* and Ab* were determined by counting and conversion of counts/min. into weights of antigen and antibody.

In the experiment recorded in Table II, the mixtures of Ag and Ab in 10 ml. of 0.9% saline solution were treated as in Table I, exp. no. 1. The supernatant was discarded. The precipitates were extracted with five 10-ml. portions of saline solution in five days (24-hr. interval) so that five washings, W-1 to W-5, were obtained. Antigen was determined by counting disintegrations, antibody in W by precipitation with trichloroacetic acid and Kjeldahl analysis of the protein precipitates. The residue, R, was weighed and its antibody content obtained by subtracting antigen from the total weight. Similar results were found when the precipitate was collected on a microfilter and repeatedly extracted in short intervals with 5-ml. portions of saline solution.

In the experiment shown in Table III, the purified γ-globulins of the pooled immune sera 114/116 were precipitated by adding 1.5 mg. of the homologous antigen (AsBG). The supernatant of the centrifuged precipitate, a, was mixed again with 1.5 mg. of antigen so that a second precipitate, b, was produced. Similarly, two further precipitates, c and d, were obtained. Each of the four precipitates was extracted 5 times with 10 ml. of saline solution as described in the preceding paragraph so that five washings, W-1 to W-5, were obtained. They were analyzed for antibody by counting. The insoluble precipitates were weighed, dissolved in 1 or 2 ml. of 1% NaOH and analyzed colorimetrically for antigen, a solution of AsBG in 1% NaOH being used as standard. Antibody weight was calculated as described in the preceding paragraph.

Dissociation of Non-radioactive Precipitates (Table IV).—Four centrifuge tubes were used in each of the experiments recorded in Table IV. Each of the four tubes contained the volume of immune serum shown in the third column of the table. The amount of antigen indicated in the table was dissolved in a small volume of 0.9% saline solution and added to each of the four tubes. The mixtures of antigen and immune serum were kept at 37° for one hour, then at −12° overnight in order to complete precipitation.[12] After thawing, mixing and centrifuging, the precipitates were washed once with 10 ml. and four times with 5 ml. of 0.9% saline solution to remove soluble serum constituents. These five washings, W-1 to W-5, were discarded. Each of the precipitates was then suspended in 35 ml. of 0.9% saline solution for equilibration. Two tubes were kept for 24 hours in the cold room, two others for 20 hours in the cold and 4 hours at room temperature (see preceding section). After centrifuging at the desired temperature, the equilibration procedure was twice repeated. In this manner three extracts, W-6 to W-8 were obtained. These washings were colorless. The precipitates were analyzed as described in the preceding paragraphs by means of gravimetry and colorimetry. Ten ml. of each of the extracts were analyzed for their protein content. In some of the experiments, 10 ml. of W-6 was mixed with 0.1 mg. of the homologous antigen, kept for 30 minutes at 37°, then at 3° overnight and centrifuged. The weight of the isolated precipitates is shown in parentheses.

Reprecipitation of Dissociated Antibody (Table V).—In 10 ml. of the pooled immune sera 290/313/316 (anti-AsBG) a precipitate was produced by the addition of 6.3 mg. of antigen, dissolved in 0.25 ml. of 0.9% saline solution. After incubation at 37° for 30 minutes the precipitate was centrifuged off and washed with 5 ml. of saline solution. It was then dissociated by suspension in 5 ml. of saline solution, incubation at 37° for 30 minutes and keeping at room temperature for 3 hours. The insoluble residue was centrifuged off and the saline extract separated. Four ml. of the saline extract was dialyzed against cold distilled water, evaporated in an evacuated desiccator over KOH pellets at room temperature and the dry residue dissolved in 1 ml. of 0.9% saline. Into each of four small tubes 0.2 ml. of the solution was placed. In two of the tubes the volume was brought to 0.8 ml. by the addition of 0.6 ml. of saline solution. Then, AsBG or AsOv, dissolved in 0.025 ml. of saline solution was added. After incubation at 37° for 30 minutes and keeping in the refrigerator for 3 days, the precipitates were centrifuged off, washed as described before, air-dried and dissolved in 3 ml. of 0.01 *N* NaOH solution. The amount of precipitated protein was calculated from the absorbancy of the solution at 283 mμ.

That complete equilibration between precipitate and supernatant solution is accomplished in our experiments is shown by the following results. Two portions, (a) and (b), of an immune serum against arsanilazo bovine γ-globulin were precipitated by adding 1.0 mg. of the homologous antigen, dissolved in 0.1 ml. of saline solution. Sample (a) was kept at 3° for 24 hours, (b) for 6 days. They were washed with two 3-ml. portions of 0.9% saline solution at 3°, immediately after the times indicated, and then analyzed. We found a total weight of (a) 6.62, (b) 6.74 mg.; antigen: (a) 0.97, (b) 0.97 mg.; calculated antibody: (a) 5.65, (b) 5.77 mg. The difference between (a) and (b) is too small to be significant. We can be sure, therefore, that equilibration takes place within less than 24 hours at 3° and more rapidly at 27°.

Results

The principal result of our investigation is the observation that antigen–antibody precipitates, on washing with 0.9% saline solution, lose significant amounts of antibody but not of antigen. This is shown quite clearly in the Tables I and II where purified antibody, free of other serum proteins, was precipitated by antigen. In another experiment (Table III) the purified γ-globulins of an immune serum were precipitated by four successive additions of the homologous antigen. The first precipitate was obtained in an excess of antibody, the last in an excess of antigen. All four precipitates lost protein on repeated extraction with saline solution. The antigen content of the extracts was too small for quantitative microcolorimetric analysis; approximate determinations showed that the Ab/Ag ratio in the extracts was much higher than in the precipitates. In the experiments recorded in Table IV whole sera were used instead of purified antibodies or immune globulins. Therefore, the first five washings, which contained other serum proteins, were discarded; only the saline extracts no. 6 to 8 were analyzed. The last column of the table shows that the antibody concentration in ex-

(12) C. G. Pope, quoted in J. R. Marrack and F. C. Smith, *Proc. Roy. Soc.* (*London*), **B106**, 1 (1930).

tract W-6 was within the same order of magnitude in all experiments, although in some of them (no. 32/33, 36/37, 34/35, and 46/49 c) the precipitates were produced by homologous antigen, in the others by heterologous antigens. In each of these experiments more antibody was extracted at the higher temperature.

The antibody nature of the extracted protein is proved by its precipitability with the homologous antigen. The low yields of precipitate recorded in Table IV are not surprising in view of (a) the very low concentration of antibody in the saline extract, (b) the dissociation of the antigen–antibody complexes in the saline solution, and (c) the instability of purified[10] antibody solutions. Table V shows that the first two of these factors are eliminated to a certain extent by reducing the volume of the antibody solutions. The yield of precipitate is increased considerably by this procedure.

TABLE I

EXTRACTION OF A PRECIPITATE FORMED BY ANTHRANILAZO BEEF SERUM PSEUDOGLOBULIN (AG) AND THE PURIFIED HOMOLOGOUS ANTIBODY (AB) WITH 10 ML. OF 0.9% SALINE SOLUTION

Expt.[a]		a	b	c	d
Temp. (±1.5°), °C.		3.5	3.5	23	23
Determined		Ag*, μg.	Ab*, μg.	Ag*, μg.	Ab*, μg.
Exp. no.[b]					
1	Supernatant	10	886	10	876
1	Precipitate	75	556	75	543
2	Supernatant	0.5	51	0.8	53
2	Precipitate	86	430	84	468
3	Supernatant	0.03	17	0.4	31
3	Precipitate	78	417	76	364

[a] In the experiments recorded in columns a and c, radioactive antigen was used, in b and d radioactive antibody. [b] In experiment no. 1 the precipitates a, b, c and d were extracted with saline solution once, in exp. no. 2 twice and in no. 3 three times (see text).

TABLE II

EXTRACTION OF A PRECIPITATE FORMED BY 1.0 MG. OF RADIOACTIVE ARSANILAZO BEEF SERUM PSEUDOGLOBULIN (AG*) AND 7.35 MG. OF THE PURIFIED HOMOLOGOUS ANTIBODY (AB) WITH 0.9% SALINE SOLUTION[a]

Temp. °C. (±1.5)	3.5° Ag*	3.5° Ab	3.5° Ag/Ab × 100	28° Ag*	28° Ab	28° Ag/Ab × 100
Washing (W-1)	7.2	495	1.5	10.5	585	1.8
Washing (W-2)	3.6	364	1.0	4.8	400	1.2
Washing (W-3)	2.6	243	1.1	3.8	285	1.3
Washing (W-4)	2.4	298	0.8	1.9	206	0.9
Washing (W-5)	1.2	185	0.6	1.9	222	0.9
Residue (R)	*530*	*2750*	*19.3*	*530*	*2220*	*23.8*
Precipitate (P)[b]	547	4335	12.6	552	3918	14.1

[a] The first supernatant was discarded. The saline volume in each of the five extractions was 10 ml. (see text). The figures of the table indicate weights in μg. [b] P = total precipitate before washing; computed by adding all values for Ag or Ab in washings and R.

Discussion

The loss of weight of the antigen–antibody precipitates on repeated washing with saline solution has been described by Heidelberger, *et al.*[13] Since small volumes of saline solution were used in their experiments, the amount of nitrogen in the precipitates was not altered appreciably by repeated washing. Larger losses were observed in the experiments of Talmage and Maurer[14] where the precipitation was carried out in a dilute solution. In all these experiments the loss of weight of the precipitates was attributed to a definite, although low, solubility of the precipitates in 0.9% saline solution. Our own experiments demonstrate, however, that the antibody/antigen ratio in the saline extracts is much higher than in the precipitates and that only negligibly small amounts of antigen pass into the saline solutions (Table I to III). The ratio Ab/Ag in the precipitates decreases continually on washing with saline solution; thus it drops in Table II at 3.5° from 4335/547 = 7.9 to 2750/530 = 5.2 after five extractions with saline solution. Similar changes are observed in all other experiments (Tables I to IV). Our findings are in agreement with earlier observations of Marrack and Hoellering[15] and Oudin and Grabar[16]; both groups of workers found preferential extraction of antibody from precipitates by saline solution.

The amount of extracted antibody increases when the temperature is raised from 3° to 25 ± 3°. The larger weight of precipitates at low temperatures has led other authors to the view that keeping at low temperatures for several days is a prerequisite for complete precipitation. Our results demonstrate, however, that the higher yield of precipitate at lower temperatures is caused by a shift in the equilibrium. If precipitates kept in the refrigerator for several days were warmed up to room temperature, they again lost the bound antibody.

The antigens used in our experiments contain approximately 20 to 30 moles of azophenylacyl groups per 10^5 gram of azoprotein. An azoprotein formed from bovine serum albumin by coupling with diazotized sulfanilic acid migrated in paper electrophoresis at *p*H 8.6 as a single band. Neither the migration velocity nor measurements of the viscosity indicate any drastic change in the shape or size of the molecules. We assume, therefore, that each of the azoprotein solutions contains molecules of a similar degree of substitution rather than a mixture of lightly and heavily substituted azoproteins, and that the molecular weight of the azoproteins is similar to that of the parent proteins.

Each of the azoproteins induces the formation of at least two, but probably more, types of antibody. One of these antibodies is adjusted to and combines with the chemospecific azophenylacyl group; an-

(13) (a) M. Heidelberger and F. E. Kendall, *J. Exp. Med.*, **62**, 697 (1935); (b) H. E. Stokinger and M. Heidelberger, *ibid.*, **66**, 251 (1937); (c) M. Heidelberger, E. A. Kabat and M. M. Mayer, *ibid.*, **75**, 35 (1942); M. Heidelberger and F. E. Kendall, *ibid.*, **55**, 555 (1932).

(14) D. W. Talmage and P. H. Maurer, *J. Inf. Dis.*, **92**, 288 (1953).

(15) J. R. Marrack and H. F. Hoellering, *Brit. J. Exp. Path.*, **19**, 424 (1938).

(16) J. Oudin and P. Grabar, *Ann. Inst. Pasteur*, **70**, 7 (1944).

TABLE III

SERIAL PRECIPITATION OF I^{131}-γ-GLOBULIN FROM THE POOLED ANTISERA 114/116 BY THE SERIAL ADDITION OF 1.5 MG. OF ARSANILAZO BEEF SERUM GLOBULIN AND EXTRACTION OF THE PRECIPITATES WITH 0.9% SALINE SOLUTION[a]

Temp., °C. (±1.5)	3				27			
Extracted ppt.	a	b	c	d	a	b	c	d
Antibody content of extracts			μg.				μg.	
W-1	657	750	470	430	920	840	655	460
W-2	363	345	289	213	545	495	504	175
W-3	257	255	201	120	432	286	220	103
W-4	240	232	163	88	333	219	134	72
W-5	220	165	106	68	239	115	95	65
Residue								
antibody (mg.)	5.70	4.15	2.49	1.47	4.76	2.76	1.67	1.11
antigen (mg.)	1.50	1.15	0.89	0.60	1.50	1.04	0.78	0.56
Ag/Ab × 100	26	28	36	41	32	38	47	50

[a] Ten ml. of saline was used for each extraction. The amount of antibody found in the extracts W-1 to W-5 was determined by measuring the radioactivity of the extracted protein; the counts/min. were converted into weights. The result is shown in the table as μg. of antibody. The residues were analyzed by gravimetry and colorimetry (see text.)

TABLE IV

DISSOCIATION OF PRECIPITATES OBTAINED FROM IMMUNE SERA IN 0.9% SALINE SOLUTION

(All figures except those marked by an asterisk are average values of duplicate analyses.)

Exp. no.	Injected antigen	Immune serum (ml.)	Test antigen Type[a]	Test antigen (mg.)	Temp., °C. (±1.5)	Antibody content of 35 ml. W-6 (mg.)[b]	W-7 (mg.)	W-8 (mg.)	Washed ppt. Total wt. (mg.)	Washed ppt. Antigen content (mg.)	− log K[c] for W-6
17/18	Su-BG	15.0	Su-Ov	3.0	2	0.81	0.23	0.03	6.53	0.84	6.84
					26	1.02	0.15	0.09	5.98	0.81	6.74
23/24	Su-Ov	12.0	Su-BG	3.0	1	0.52	0.52	0.16	2.60	0.48	7.03
					23	0.57	0.18	0.13	2.49	0.47	6.99
32/33	Su-Ov	11.0	Su-Ov	2.0	4	1.53	1.01	0.40	15.92	1.44	6.56
					29	2.25(0.12)	1.38	0.55	15.10	1.30	6.40
36/37	Su-BG	6.5	Su-BG	2.0	5	1.07(0.50)	0.90	0.69	8.02	1.06	6.72
					24	1.38(0.72)	0.93	0.74	7.56	1.08	6.61
34/35a	As-BG	7.0	As-BG	2.0	5	3.74(1.20)	1.61	0.73	14.25	1.28	6.17
					24	5.06(1.64)	2.10	1.25	12.42	1.21	6.05
34/35b	As-BG	7.0	As-BG	4.0	5	3.82(0.92)	1.82	1.00	25.27	2.66	6.17
					24	5.15(1.30)	2.51	1.52	22.29	2.60	6.04
46/49a	As-BG	8.0	As-Ov	1.5	1	0.39*	0.34*	0.18*	6.74	0.83*	7.16
					23	0.63*	0.55*	0.28*	5.78	0.79*	6.95
46/49b	As-BG	8.0	BG	1.5	5	1.39	0.42	0.29	4.50	..	6.60
					24	1.71	0.46	0.29	3.48	..	6.51
46/49c	As-BG	8.0	As-BG	1.5	2	2.00*	0.60	0.85	10.61	1.22	6.44
					25	2.27*	1.75	1.09	9.75	1.08	6.39

[a] Su, sulfanilazo; As, arsanilazo; BG, beef serum pseudoglobin; Ov, ovalbumin. [b] In brackets antibody precipitated by adding 0.1 mg. of antigen to aliquots of W-6. [c] Calculated for a molecular weight of 160,000 of antibody.

other type combines with the unknown species-specific groups of the antigen molecule; a third type of antibody may combine with an area of the antigen molecule which contains both of these groups.[17] Thus in exp. no. 46/49 (Table IV) only some of the antibodies are precipitated by AsOv or by BG; much more precipitate is produced by adding the homologous antigen, AsBG. It is obvious that in this case the combining sites on the surface of the antigen molecules are not identical and that we are dealing with a mixture of antibodies of different specificities.

Since the antigen–antibody complex, according to our observations, dissociates markedly in saline solution, it is impossible to precipitate all of the antibody at the equivalence point. This difficulty cannot be overcome by adding more antigen because soluble antigen–antibody complexes are formed under these conditions. The question may be raised, therefore, whether all of the protein extracted from the precipitates by saline solution is antibody, or whether it contains other proteins such

(17) F. Haurowitz and P. Schwerin, *Brit. J. Exp. Path.*, **23**, 146 (1942).

as complement. The presence of active complement is excluded because all sera were inactivated at 55°. Although small amounts of inactivated complement or of other proteins can be adsorbed non-specifically to antigen–antibody precipitates, such proteins would be removed by the purification procedure in which antibody is extracted by 0.1 N HCl; insoluble material is centrifuged off after neutralization of the acid antibody solution. We have all reason, therefore, to assume that the solutions of purified antibody are free of complement and of other, easily adsorbable, proteins. This view is supported by the observation that results obtained with γ-globulins (Table III) or with whole immune sera (Table IV) were identical with those obtained with purified antibody solutions (Table I and II). We cannot exclude, however, in any of our experiments the presence of "low-grade" antibody, *i.e.*, of antibody which forms soluble antigen–antibody complexes, but is co-precipitated in the presence of true, precipitating, antibody.[13]

TABLE V

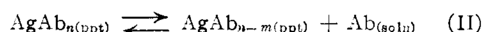
INFLUENCE OF ANTIBODY CONCENTRATION ON THE YIELD OF PRECIPITATE[a]

Experiment	a	b	c	d
Anti-AsBG (ml.)	0.2	0.2	0.2	0.2
0.9% NaCl (ml.)	0	0.6	0	0.6
Added AsBG (mg.)	0.05	0.05	0	0
Added AsOv (mg.)	0	0	0.05	0.05
Precipitate (mg.)	0.350	0.165	0.105	0.060
D (mg.)[b]	0.185		0.046	

[a] Anti-AsBG was prepared by precipitating arsanilazo beef serum γ-globulin (AsBG) with the homologous immune serum, extraction of antibody from the washed precipitate by saline, dialysis and concentration of the antibody solution from 0.8 to 0.2 ml. (see text). AsOv, arsanilazo ovalbumin. [b] D is the increase in mg. of precipitate accomplished by reducing the volume of the antibody solution from 0.8 to 0.2 ml.

It is important, before further discussion of reaction I, to decide whether it represents a reversible reaction. Talmage, *et al.*,[18] proved reversibility in other antigen-antibody systems by the exchange of bound antibody with added radioactive antibody. Reversibility of reaction I is indicated by the solubility of the antigen–antibody precipitates in an excess of antigen, by the rapid release of antibody from the precipitates when the suspension of the precipitate in saline solution is warmed up from 3° to room temperature, and by the reprecipitation of the extracted antibody on addition of antigen under suitable conditions (Table V). Reversibility of the reaction is also indicated by the fact that the amount of extracted antibody depends only on the final temperature, not on the temperatures to which the system has been exposed temporarily. Thus, some of the reactions were allowed to take place at 37°; the suspensions were then frozen[12] and finally warmed up to the desired temperature and centrifuged. The results in these experiments were, essentially, identical with those in experiments where the suspensions were kept for 20 hours at 3° and for 4 hours at the final temperature. It would be desirable to prove reversibility more rigorously by the recombination of antibody with the precipitate from which it was extracted. This is not possible because the diluted solutions of purified antibody are extremely unstable. In contrast to the original immune sera, which can be repeatedly frozen and thawed without significant loss of precipitating activity, the highly diluted solutions of purified antibody lose rapidly their ability to precipitate antigen when they are kept at room temperature or in the refrigerator over periods of more than a few hours or when they are frozen and thawed.

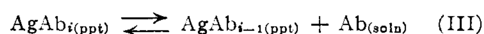
Evaluation of the Tables I to IV reveals that the ratios y/x vary from 7 to 15. Accordingly, the precipitates consist of clusters of antigen–antibody complexes, each of these formed by one Ag molecule and 7 to 15 Ab molecules. The clusters may be linked to each other by non-specific attraction between Ab molecules (Fig. 1a), by non-specific attraction between adjacent Ag and univalent Ab molecules (Fig. 1b), or by specific bonds between Ag molecules and uni- or bivalent Ab molecules (Fig. 1c). It is evident from Fig. 1 that in all three cases most of the Ab molecules are held in the lattice by only *one* specific combining group even if they are bivalent and have two specific combining groups. Other authors[6,19] already have pointed out that the "effective valence" of most of the antibodies is one. If the precipitate contained a mixture of uni- and bivalent antibody molecules, those with an effective valence of one might be extracted preferentially by saline solution. This would cause conversion of structures a and b into structure c (Fig. 1). In the following discussion only dissociation of precipitates of the type a will be considered, *i.e.*, dissociation of Ab molecules which are bound in the lattice by only one specific Ag–Ab bond. Since each of the antigen–antibody clusters contains only one Ag molecule, the dissociation of Ab molecules is represented by equation II



$$\mathrm{AgAb}_{n(\mathrm{ppt})} \rightleftharpoons \mathrm{AgAb}_{n-m(\mathrm{ppt})} + \mathrm{Ab}_{(\mathrm{soln})} \quad \text{(II)}$$

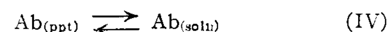
where n is the maximum number of Ab molecules bound to one Ag molecule.

If we assume that the dissociation of the AgAb_n clusters takes place stepwise, one Ab molecule being removed from the AgAb_n complex at a time, each step of this reaction is represented by equation III



$$\mathrm{AgAb}_{i(\mathrm{ppt})} \rightleftharpoons \mathrm{AgAb}_{i-1(\mathrm{ppt})} + \mathrm{Ab}_{(\mathrm{soln})} \quad \text{(III)}$$

where i is equal to or smaller than n. In view of the fact that both AgAb_i and AgAb_{i-1} are insoluble and have unit activity, equation III can be replaced by the simpler equation IV (suggested by the Referee).



$$\mathrm{Ab}_{(\mathrm{ppt})} \rightleftharpoons \mathrm{Ab}_{(\mathrm{soln})} \quad \text{(IV)}$$

Since [Ab], the molar concentration of antibody is less than 10^{-5} the difference between concentration and activity is probably small and can be neglected.

(18) D. W. Talmage, H. R. Baker and W. Akeson, *J. Inf. Dis.*, **94**, 199 (1954).

(19) M. F. Morales, J. Botts and T. L. Hill, THIS JOURNAL, **70**, 2339 (1948).

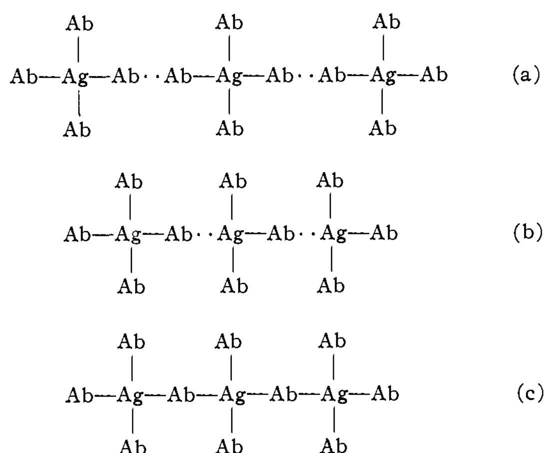


Fig. 1.—Antigen–antibody complexes. Specific bonds between antigen (Ag) and antibody (Ab) molecules are indicated by solid lines, non-specific bonds by dotted lines.

Both equations III and IV lead to the results that K_A, the affinity constant, is equal to 1/[Ab].

Our experimental data allow us to calculate i for each phase of the extraction with saline solution. Thus, in experiment a of Table III at 3° the following weights of antibody were found before extraction and after 1, 2, 3, 4 and 5 extractions with saline solution: 7.44, 6.78, 6.42, 6.16, 5.92 and 5.70 mg. The corresponding Ab/Ag ratios in the precipitates are: 5.0, 4.5, 4.3, 4.1, 3.9 and 3.8. Since the molecular weight of the antibody is close to that of the azoprotein used, the molar ratio, i, in the precipitates is identical with the weight ratio. If the antibody concentrations calculated from Table III are converted into affinity constants, K_A, and the values of K_A are plotted against i, the curves of Fig. 2 are obtained. Evaluation of Tables II and IV leads to similar results.

Although the combination of Ab with the $AgAb_{i-1}$ complex is similar to adsorption, the process differs from typical adsorption phenomena by the composition of the solid phase which contains from 60 to 90% of the adsorbed material, Ab. Similar high Ab/Ag ratios are found also in those instances where both antigen as well as antibody are proteins of the same type, *e.g.*, when the antigen is bovine or ovine serum globulin and the antibody rabbit serum globulin. This high Ab/Ag ratio is in excellent agreement with the multivalence of Ag and the presence of only one, possibly two, combining groups in Ab molecules, shown in Fig. 1. If the combination of an Ab molecule with a vacant site on the surface of the cluster $AgAb_{i-1}$ were directed merely by statistical distribution,[6,19] the ratio of the affinity constants K_p/K_q for the complexes $AgAb_p$ and $AgAb_q$ would be

$$K_p/K_q = q(n - p + 1)/p(n - q + 1) \qquad \text{(V)}$$

where n is the maximum number of Ab molecules per Ag molecule, and p and q are values of i smaller than n but larger than 1. Using equation V we find the ratios K_1/K_2, K_2/K_3 and K_3/K_4 for $i = 5$ as 2.5, 2.0 and 2.0, respectively; K_1/K_3 is 5. If i = 6 or 7, K_1/K_3 decreases to 4.5 and 4.2, respectively. The experimental values for K_1/K_2, K_2/K_3 and K_3/K_4 are close to 3.3 for each of these ratios; K_1/K_3 is approximately 12, considerably higher than the calculated value of 5 or less. The deviation from purely statistical distribution is not surprising in view of (1) the presence of two types of bonds between Ag and Ab (Fig. 1), and (2) the heterogeneity of the antibody population proved by exp. no. 46/49 (Table IV). Evidently, these factors are superimposed on statistical effects and cause the discrepancy between the calculated and the experimental values.

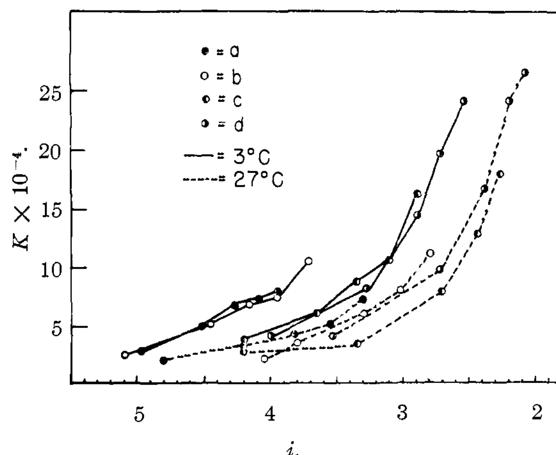


Fig. 2.—Dissociation of antigen–antibody precipitates. The values for i, the Ab/Ag ratio, and for K, the affinity constant, are calculated from the data of Table III.

An attempt has been made to estimate thermodynamical constants for reaction III or IV. Obviously, this is permissible only when the formation of $AgAb_i$ from $AgAb_{i-1}$ and Ab is a reversible reaction. The evidence for reversibility has been discussed in preceding paragraphs. It also has been pointed out that our affinity constants refer only to antigen–antibody complexes of the type a (Fig. 1), not to those of type c where we are dealing with a more complicated process which involves bivalency of some of the Ab molecules. If the free energy of combination of Ab with vacant sites on the surface of the complex $AgAb_{i-1}$ is computed from K_A, ΔF values which vary from −8 to −10 kcal. per mole of Ab are obtained for the systems recorded in Tables I–IV. The ΔH values estimated from the affinity constants measured at two different temperatures vary from −3.2 to −7.5 kcal. per mole of antibody. The numerical values of ΔH are higher in the equivalence zone (Fig. 2b) than in an excess of antibody, a, or antigen, c and d. Our values for ΔH are considerably lower than those found by Wurmser and Filitti-Wurmser[20] for the combination of isoagglutinins with red blood cells, and by Boyd, *et al.*,[21] for the combination of hemocyanin with antihemocyanin. Both groups of authors investigated the combination of free Ab

(20) R. Wurmser and S. Filitti-Wurmser, *Biochim. Biophys. Acta*, **4**, 238 (1950).

(21) W. C. Boyd, J. B. Conn, D. C. Gregg, G. B. Kistiakowsky and R. M. Roberts, *J. Biol. Chem.*, **139**, 787 (1941).

molecules with free Ag molecules, whereas we investigated the combination of Ab with the $AgAb_{i-1}$ complex. Hence our ΔH values do not include the enthalpy of formation of $AgAb_{i-1}$ from its components, and are therefore lower. In all of our experiments we find an increase in entropy on combination of Ab with $AgAb_{i-1}$. This is in agreement with previous results[8] and with reports of Doty and Epstein[22] on the combination of antibody with haptens. In the experiment recorded in Table III, ΔS varied from 5 to 9 e.u. in the equivalence zone, and from 12 to 20 e.u. at other Ab/Ag ratios. The positive value of ΔS may be due to the release of water molecules from the combining sites of Ag and Ab,[8,23] or to the loosening of the structure of $AgAb_{i-1}$ when it combines with Ab to form $AgAb_i$.

BLOOMINGTON, INDIANA

(22) P. Doty and S. I. Epstein, *Nature*, **174**, 89 (1954).

(23) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948).