STUDIES ON NON-PRECIPITATING ANTIBODY

I. The Conversion of Non-Precipitating Antibody to a Precipitin-Like Material in Vitro*

By LUDWIG A. STERNBERGER, M.D., SAMUEL M. FEINBERG, M.D., AND MARTHA E. CLARKE

(*From the Allergy Research Laboratory, Department of Medicine, Northwestern University Medical School, Chicago*)

In a previous paper (1) we described the appearance of a soluble circulating antibody-antigen complex in rabbits after single intravenous injections of large doses of bovine plasma albumin or bovine plasma gamma globulin, or after intramuscular injections of these agents with Freund's adjuvants. The soluble complex was demonstrated in the serum by brief exposure to alkali in the cold followed by neutralization and incubation. Upon this treatment precipitates separated spontaneously in some immune sera but not in normal serum.[1] The specificity of the precipitates was demonstrated by resuspension in an excess of specific antigen; the precipitates were soluble in specific antigen but not in non-specific protein (Fig. 1).

It was puzzling that large amounts of antigen and antibody could coexist in circulation without precipitating. The solubility of the antibody-antigen complex did not depend upon the solubilizing effect of excess antigen on precipitating antibody, since the complex was found in serum obtained early in immunization, while excess antigen prevailed in circulation, as well as in later phases of immunization when free antigen had disappeared from circulation and excess of free antibody was present or absent. The failure of the complex to precipitate in circulation appeared to result, therefore, from the nature of either its antibody or its antigen component. The present paper will show that the antibody of the complex is non-precipitating antibody.

The procedure used for demonstration of non-precipitating antibody was suggested by the following consideration: If soluble antibody-antigen complex is derived from a combination of antigen and non-precipitating antibody *in vivo* some sera may be found to possess non-precipitating antibody in excess of that

[1] For illustration see Fig. 1 in reference 22.

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necessary to bind all the antigen. Such uncombined antibody would not precipitate upon addition of antigen to the original serum. Since the alkali treatment converts non-precipitating antibody-antigen complex to a precipitating form there may be a likelihood that it would also convert non-precipitating uncombined antibody to precipitating uncombined antibody. The uncombined non-precipitating antibody might, therefore, precipitate if antigen were added to the treated serum.

saline   1:6.7 BPγG   1:6.7 CBPA   1:6.7 BPA
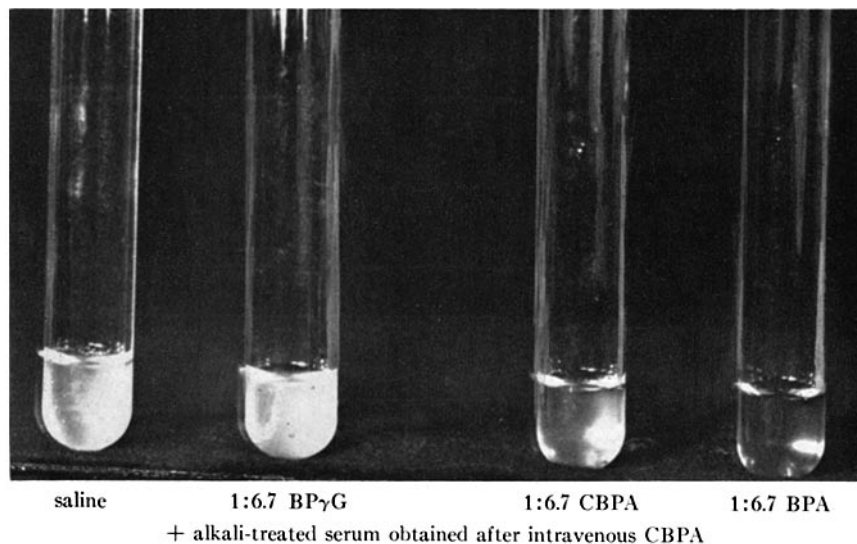+ alkali-treated serum obtained after intravenous CBPA

Fig. 1. Test for specificity. Rabbit blood was obtained after immunization with CBPA. The serum was treated with alkali and antibody-antigen complex allowed to separate for one day at 1°C. It was then resuspended and mixed with an equal volume of 0.85 per cent sodium chloride solution containing merthiolate 1:10,000 and with a 1:6.7 solution of BPγG, CBPA, and powdered BPA respectively.

Materials and Methods

Antisera.—Flemish Cross giant male rabbits 6 months old were immunized by biweekly or triweekly intravenous injections of 0.1 gm./kg. of crystalline bovine plasma albumin (CBPA)[2] or bovine plasma gamma globulin (BPγG)[3] or by a single intramuscular injection of 80 mg. of these agents or of 80 mg. of powdered bovine plasma albumin with Freund's complete adjuvants (2, 3). Blood was obtained in 60 ml. amounts by ear artery puncture, the serum separated under aseptic conditions and merthiolate® added to a concentration of 1:10,000. The sera from rabbits receiving intravenous injections of antigen were obtained 2 weeks after each injection, quick frozen at about −60°C. and stored at about −23°C. Immediately before use these sera were thawed at 37°, pooled, and kept at 1°C. The sera from rabbits immunized with Freund's adjuvants were obtained in weekly or biweekly bleedings of 60 ml. each between the 28th and the 322nd day after immunization. Following addition of merthiolate® they

[2] CBPA, crystalline bovine plasma albumin.
[3] BPγG, bovine plasma gamma globulin.

were stored at 1°C. under aseptic conditions. At irregular intervals the sera were pooled and either lyophilized or Seitz-filtered.

Antigens.—Crystalline bovine plasma albumin (CBPA) and bovine plasma gamma globulin (BPγG) were obtained from Armour and Company, Chicago. Crystalline egg albumin was prepared by the method of Keckwick and Cannan (4), quick frozen at −60°C., and stored at −23°C. All test antigens were diluted in 0.16 N sodium chloride solution containing merthiolate® 1:10,000. Their protein contents were determined with the Folin-Ciocalteu reagent by the method of Heidelberger and McPherson (5) and the nitrogen equivalent of the optical densities encountered at various concentrations was established by comparison with nesslerization (6).

Treatment of Serum with Alkali.—As described previously (1) sodium hydroxide solution was added to the serum at 1°C. until the pH read 12.7. (This corresponds to a pH of 12.6 if the combined error of temperature adjustment and sodium ion concentration is taken into account. Much less alkali is needed than at room temperature and it is not implied that a true pH of 12.6 is attained. For details of pH adjustment see reference 7). After 6 minutes of exposure to alkali the serum was neutralized to the original pH. The serum was not incubated as described previously at 56°C. for 30 minutes before alkali treatment, since heat inactivation had no discernible effect on the determination of circulating antibody-antigen complex by precipitation and since no complement fixation tests were done in the present work.

Estimation of Antibody-Antigen Complex.—4 ml. triplicate portions of alkali-treated sera or diluted untreated controls were left at 1° for 11 days. (Incubation at 37°C. for 2 hours as previously reported (1) was omitted). The precipitates that formed were centrifuged at 1000 g 1°C. for 20 minutes and washed 3 times with 6.0 ml. portions of 0.16 N sodium chloride solution at 1°C. Their protein contents were determined by the method of Heidelberger and McPherson (5).

Reaction of Antibody and Antigen in Alkali-Treated Sera.—4 ml. portions of supernates from the tubes with alkali-treated sera and from untreated controls were added in duplicates or triplicates to 1.0 ml. portions of various concentrations of antigen in 0.16 N sodium chloride solution containing merthiolate 1:10,000. The tubes were left at 1°C. for 11 days and any precipitates were centrifuged, washed, and estimated as described for antibody-antigen complex.

Radioiodination of Antigen.—Antigen was labelled with I¹³¹ by the method of Gilmore and coworkers (8) with the following modifications: For the iodination of each gram of protein only 4.34 mg. of carrier potassium iodide and 0.2 gm. ammonium persulfate were used. The ammonium persulfate solution was brought to pH 7.6 with sodium hydroxide as suggested by Radin (9). 94 per cent of the labelled BPγG and 100 per cent of the labelled CBPA were precipitable by specific antiserum.

Estimation of Antigen.—In some experiments free antigen was determined by the method of Heidelberger and Kendall (10) which involves the addition of an excess of calibrated antiserum to an unknown amount of antigen. Determinations were made in duplicates or triplicates on the supernates remaining after addition of antigen to alkali-treated or untreated sera. In other experiments I¹³¹-labelled antigen was estimated in a well scintillation counter on 2 ml. samples of washed duplicate antibody-antigen precipitates dissolved in 2.2 ml. of 5 N sodium hydroxide.

Agar Diffusion Studies.—Serum agar tubes were set up by the method of Oudin (11) as described by Vaughan and Kabat (12). The tubes of experiments with CBPA as antigen were kept at 1°C. and those of experiments with BPγG at 23°C.

EXPERIMENTS AND RESULTS

Experiment 1. Precipitates Obtained from Mixtures of Antigen and Antiserum Treated with Alkali after Removal of Precipitating Antibody.—Antiserum obtained

from rabbits injected intravenously with CBPA was mixed with varying quantities of antigen and the precipitates that formed were removed. Treatment of the supernates with alkali resulted in conversion of non-precipitating to precipitating antibody-antigen complex. It will be seen that more antibody-antigen complex was precipitated from serum samples mixed with antigen than from controls mixed with saline.

Serum was obtained on day 49 from a rabbit that had received intravenous injections of 0.1 mg./kg. of CBPA on days 0, 14, and 35. The serum was divided into 4 portions. An equal

TABLE I

Precipitates Formed in Alkali-Treated Supernates Obtained from Mixtures of Anti-CBPA and Varying Amounts of Antigen

(1) μg, CBPA N added/ml. serum	(2) μg. N precipitated/ ml. serum	(3) Tests on supernates	(4) Bands precipitated by diffusion of 4 per cent CBPA through increasing dilutions of supernates in agar	(5) μg. N/ml. undiluted serum spontaneously precipitated from untreated supernates	(6) μg. N/ml. undiluted serum spontaneously precipitated from alkali-treated supernates	(7) Increase due to antigen in μg N/ml. undiluted serum precipitated spontaneously from alkali-treated supernates	(8) Tests on supernates from precipitates in column 6	(9) Bands precipitated by diffusion of 4 per cent CBPA through increasing dilutions in agar of supernates from precipitates of column 6
0	0		4‡	0	211*			16‡
5	49	Excess antibody	4	0	306	95	Excess antibody	18
72	42	Excess antigen	No band	0	404	193	Excess antibody and antigen	32
720	8	Excess antigen	No band	0	559	348	Excess antigen	No band

* This precipitate may contain some complement-like material. The other precipitates obtained after alkali treatment are presumably free of complement since they are derived from serum portions pretreated with antigen.

‡ Each number represents one band and indicates the highest dilution of serum before admixture with agar at which the band was visible.

volume of merthiolated saline was added to the first portion and equal volumes of CBPA solution containing 5, 72, and 720 μg. of antigen nitrogen per ml. were added to the succeeding portions. All mixtures were incubated and stored and precipitates determined as previously described. Each of the supernates was divided into 2 portions. One of each of these 2 portions was treated with alkali and made up to 4 volumes with respect to the original serum. The other was not treated but simply diluted to 4 volumes. Any precipitates that formed upon storage of 4 ml. samples of these treated and untreated portions were separated by centrifugation and a new set of supernates was obtained. The protein content of the washed precipitates was estimated (Table I, columns 5 and 6). The new supernates were tested for the presence of remaining antigen and antibody (columns 3 and 8) and for the capacity of forming bands of precipitation in agar tubes upon diffusion of 4 per cent CBPA by the Oudin technique (columns 4 and 9).

Table I, column 2, gives the amounts of precipitate obtained upon addition of varying quantities of antigen to the serum. Tests on the resulting supernates (columns 3 and 4) show that 5 μg. antigen nitrogen per ml. of serum left precipitating antibody in solution, while 72 and 720 μg. antigen nitrogen per ml. left antigen but no precipitating antibody. Simple dilution and storage of the supernates failed to produce additional precipitation (column 5). However, when these supernates were treated with alkali large amounts of precipitate were obtained (column 6) indicating the presence of antibody-antigen complex. The precipitate from the treated supernate of the serum sample to which no antigen was added reflects the contents of antibody-antigen complex of the original serum. Addition of antigen to the serum followed by removal of precipitating antibody increased the amount of non-precipitating antibody-antigen complex disclosed by precipitation after alkali treatment.

Gel diffusion analysis suggested that the antibody disclosed in the untreated or alkali-treated serum reacted with a single component of the antigen (columns 4 and 9). After addition of various quantities of antigen and removal of the ensuing precipitates, the untreated supernates contained either excess of antigen or excess of antibody but not both (column 3). Thus, following addition of 72 μg. CBPA nitrogen per ml. to the untreated serum, the supernate contained excess of antigen only. However, when this supernate was treated by alkali and antibody-antigen complex allowed to separate, the new supernate contained both, excess of antigen as well as excess of material giving a band of precipitation upon gel diffusion (column 8). Inhibition by antigen excess of precipitation of the material disclosed by alkali treatment was not apparent in the present experiment. However, in other experiments such inhibition was obtained with larger amounts of antigen (3 to 25 mg. nitrogen per ml. undiluted serum).

There may be two reasons for the increase of precipitation in alkali-treated serum samples depleted to varying degrees of precipitating antibody by the addition of antigen as compared to samples to which no antigen was added. Either part of the antigen may have aggregated with the antibody-antigen complex existing in the serum or antigen may have caused precipitation of another soluble serum factor after alkali treatment. To clarify this point the following experiment was performed.

Experiment 2. Conversion of a Non-Precipitating Serum Factor to a Precipitin-Like Material in Serum Depleted of Precipitating Antibody and of Antibody-Antigen Complex.—The procedure is illustrated diagrammatically in Fig. 2. Serum is depleted of precipitating antibody by addition of a sufficient amount of antigen (a single addition of antigen in equivalence or a series of additions of antigen in antibody excess is satisfactory). The precipitate composed of precipitating antibody and antigen is removed (Fig. 2, tube 2). The supernate,

now devoid of precipitating antibody (tube 3), is treated by alkali. A precipitate composed of antibody-antigen complex forms (tube 4) and is removed. When specific antigen is added to the supernate (tube 5) a precipitate forms (tube 6). This is a specific precipitate since none forms upon addition of a non-specific protein, as illustrated by tube 7. Serum depleted of precipitating antibody (tube 8), which is not treated by alkali, forms no spontaneous precipitate
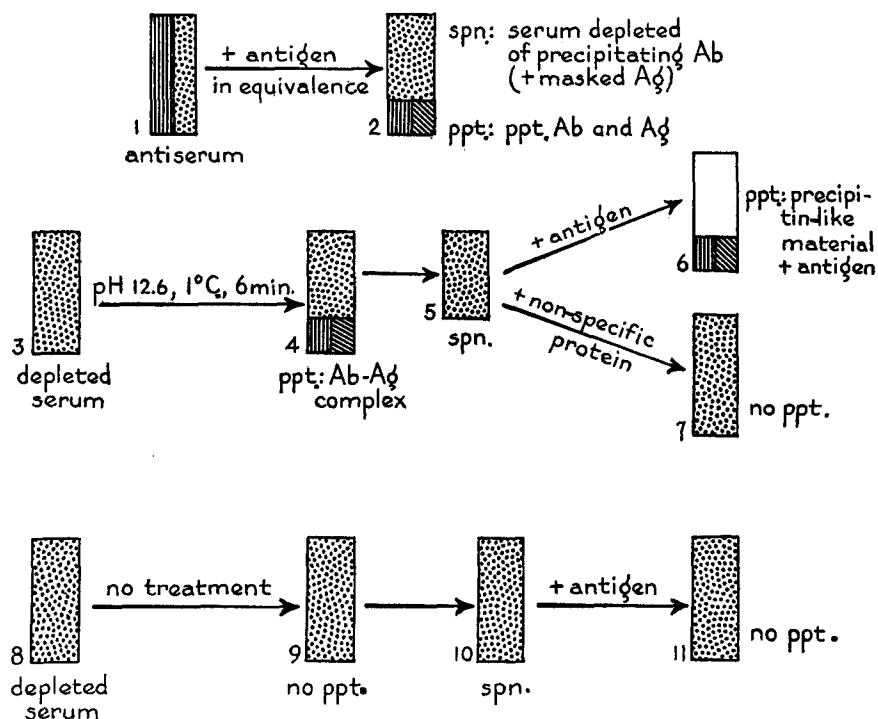

FIG. 2. Schematic representation of estimation of non-precipitating antibody in serum following depletion of precipitating antibody, alkali treatment, and removal of antibody-antigen complex.

(tube 9). Additional treatment of this serum with specific antigen does not produce a precipitate either (tube 11). The following is a detailed description of such an experiment.

A pool of sera from rabbits which had received 2 biweekly intravenous injections of 0.1 mg./kg. of CBPA was obtained 2 weeks after the second injection. Two derivatives from this pool were used. One, serum A, was depleted of precipitating antibody only, and the other, serum B, was depleted of precipitating as well as coprecipitating antibody ("monovalent" antibody of Heidelberger (13)). Non-depleted serum was also used in some phases of the experiment.

Serum A was prepared by 2 additions of 1.4 μg. CBPA per ml. (first admixture at 37°C. for 2 hours, followed by centrifugation at 1000 g at 1°C. for 20 minutes, second absorption at 37° for 2 hours, followed by storage at 1°C. for 11 days and centrifugation at 1000 g at 1°C. for 40 minutes). After the two treatments with antigen the serum failed to form precipitates with varying quantities of antigen in a qualitative test involving incubation for 2 hours at 37° and 1 day at 1°C.

Serum B was prepared like serum A but in addition was depleted of coprecipitating antibody with a potent anti-BPA rabbit serum produced with Freund's adjuvants and an amount of antigen known to precipitate with this antiserum in slight antigen excess. The protocol

TABLE II

Estimation of Coprecipitating Antibody in Anti-CBPA Depleted of Precipitating Antibody

	(1)	(2)	(3)
Precipitating rabbit anti-BPA pool, *ml*	0.1	0.1	0.1
Normal rabbit serum, *ml*	1.0	—	—
Merthiolated saline, *ml*	0.2	—	—
Rabbit anti-CBPA depleted of precipitating antibody, *ml*. (dilution 1:1.2)	—	1.2	—
CBPA, 18 μg. N/ml., *ml*	1.0	1.0	1.0
Pooled supernate from column 2, *ml*	—	—	2.3
Nitrogen precipitated, μg	105*	126‡	102§
Antigen in supernate, μg	3‖	0‖	Excess antigen¶
Coprecipitating antibody, μg		18	

All tubes were incubated at 37° for 2 hours and left at 1° for 11 days. Sera were separated by centrifugation at 1000 g 1° for 20 minutes. Column 3 was set up in order to show whether the removal of coprecipitating antibody in column 2 was complete.

* Average of septet determinations.

‡ Average of quadruplicate determinations.

§ Average of triplicate determinations.

‖ Estimated with calibrated antiserum.

¶ Qualitative estimation only.

for this procedure is given in Table II. Each milliliter of serum depleted of precipitating antibody contained 18 μg. of coprecipitating antibody nitrogen.

Non-depleted serum, sera A and B were divided into two portions each. One of each was treated with alkali to precipitate antibody-antigen complex and made up with merthiolated saline to 4 volumes with respect to the original non-depleted serum. The other of each portion (control) was merely diluted to 4 volumes with merthiolated saline. The resulting six serum preparations were kept at 1°C. for 11 days and centrifuged at 1000 g at 1°C. for 40 minutes. This removed the antibody-antigen complex, disclosed in the three treated serum preparations. Its quantity was estimated from the washed precipitates obtained from aliquot triplicate samples of 4 ml. each of the six serum preparations (Table III).

The number of participating antibody-antigen systems remaining in the supernates of the six serum preparations was determined from the number of bands formed upon diffusion of a 4 per cent solution of CBPA into mixtures of the serum preparations with agar (Table IV).

The supernates of the following 4 of the 6 serum preparations described were used to es-

tablish the amount of material precipitating with antigen from alkali-treated serum after removal of precipitating antibody: The supernates obtained after allowing antibody-antigen complex to separate from alkali-treated serum A and B and, as controls, the supernates from the diluted untreated portions of serum A and B. Four ml. duplicate samples of the supernates were added to 1.0 ml. volumes of varying quantities of CBPA or merthiolated saline or crystal-

TABLE III

Antibody-Antigen Complex in Non-Depleted and Depleted Anti-CBPA Serum

	μg. antibody-antigen complex N precipitated/ml. undiluted serum
Non-depleted serum...	120*
Serum depleted of precipitating antibody (serum A)...................	169
Serum depleted of precipitating and coprecipitating antibody (serum B)..	253‡

* This precipitate may contain some complement-like material. The other precipitates are presumably free of complement since they are derived from serum pretreated with antigen.

‡ Only an insignificant trace of antibody-antigen complex was found in the supernate of the serum antigen mixture used for removal of coprecipitating antibody.

TABLE IV

Number of Participating Antibody-Antigen Systems in Alkali-Treated and Untreated Anti-CBPA

Serum	Bands precipitated by diffusion of 4 per cent CBPA through mixtures of equal parts of agar and a 1:4 dilution of serum	
	Untreated	Alkali-treated
Non depleted.................................	2.0 H*	2.0 MH
Depleted of precipitating antibody (serum A)....	No band	2.1 M
Depleted of precipitating and coprecipitating antibody (serum B).........................	1.1 LM, 1.0 M	1.6 MH, 1.0 L, 0.9 L
Supernate from normal serum mixed with antigen and antiserum used for removal of coprecipitating antibody.................................	1.2 LM, 1.0 M	1.2 LM, 1.0 M

* Each figure indicates for one band the slope of the line obtained by plotting mobility against the square root of time. Letters indicate the intensity of each band (H—heavy; MH—medium to heavy; M—medium; LM—light to medium; L—light; LL—faint). The number of bands is indicated by the number of figure-letter entries.

line egg albumin. The tubes were stored in the usual way and centrifuged. A new set of supernates was collected and the N content of the washed precipitates was determined (Table V). The supernates were treated with an excess of calibrated antiserum to determine the amount of remaining antigen (antigen removal capacity of the precipitates). Other portions of the supernates were used in gel diffusion tubes with a 4 per cent solution of CBPA as diffusing agent in order to determine the number of antibody-antigen systems remaining after precipitation with varying quantities of antigen.

Table V, column 2, shows that serum A, apparently depleted of precipitating antibody by 2 serial additions of antigen still contained a trace of precipitating antibody, too small to give a band with the agar diffusion technique (Table IV). When serum A was treated with alkali and antibody-antigen complex allowed to separate, the supernates precipitated large amounts of protein upon addition of varying quantities of CBPA (Table V, column 3). Column 4 shows that in serum B depletion of precipitating and coprecipitating antibody completely

TABLE V

Demonstration of Material Precipitable with CPBA in Antisera Depleted of Precipitating Antibody, Coprecipitating Antibody, and Soluble Antibody-Antigen Complex

Serum A, anti-CPBA depleted of precipitating antibody; Serum B, anti-CBPA depleted of precipitating and coprecipitating antibody

Antigen N added/ml. undiluted serum	μg. N precipitated/ml. undiluted serum				μg. residual CBPA N in supernate*				Residual antibody in supernate (bands precipitated by diffusion of 4 per cent CBPA through mixture of equal parts of supernate and agar)	
	Serum A		Serum B		Serum A		Serum B		Serum A	
	Un-treated	Alkali-treated	Un-treated	Alkali-treated	Un-treated	Alkali-treated	Un-treated	Alkali-treated	Untreated	Alkali-treated
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
4 μg. CBPA	0	105	0	78	0	0	3	0	No band	1.9 M‡
7 " "	0	153	0		1	0	6		" "	No band
14 " "	18	281	0	142	5	2	12	5	" "	" "
18 " "	24	293	0	142	8	5	18	10	" "	" "
36 " "	24	349	0	172	30	15	45	25	" "	" "
72 " "	12	349	0	228	100	67	82	67	" "	" "
144 " "	0	349	0	228	200	150	175	150	" "	" "
180 " "		308		222				175		
18 " crystalline egg albumin	0	0	0	0					" "	2.3 M

* Equivalent to total supernate from 1 ml. undiluted serum.

‡ See footnote of Table IV.

abolished the precipitating capacity of the serum with any amount of antigen tested. When serum B was treated with alkali and antibody-antigen complex allowed to separate, again large amounts of precipitate were obtained upon addition of CBPA to the supernates (column 5). With these data inhibition by excess antigen was not definite. Other experiments indicate, however, that inhibition can be obtained with amounts of antigen varying between 3 and 25 mg. nitrogen per ml. undiluted serum. None of the alkali-treated serum preparations gave a precipitate upon addition of the non-specific material, crystalline egg albumin.

Determination of antigen remaining in the solution after separation of the precipitates listed in columns 2 to 5 gave lower values for the alkali-treated than the untreated serum preparations (columns 6 to 9), showing that the precipitates obtained with antigen from the treated serum preparations removed antigen from solution. These data do not permit, however, a quantitative evaluation of the amount of antigen included in the precipitates since the amount of antigen recovered from untreated serum A (column 6) did not correspond very well to the amount of antigen originally added (column 1). Better agreement between these two sets of values was obtained with the untreated serum B (column 8).

Perhaps a soluble complex formed by a factor of the untreated serum A and added antigen interfered with subsequent complete precipitation of the antigen by an excess of calibrated antiserum. Employing radioiodinated antigen, such observations have previously been made by Robert Feinberg (14). For the preparation of serum B, depleted of coprecipitating as well as of precipitating antibody, more antigen was used than for the preparation of serum A. As shown below, this increased the amount of non-precipitating antibody-antigen complex (Table III) which may be capable of interference with precipitation of the antigen by subsequently added precipitating antiserum. Perhaps in the preparation of serum B (but not in the preparation of serum A) the serum factor which forms such an "interfering" complex may have been exhausted. Any further addition of antigen (as done by adding the amounts of antigen listed in column 1 to the non-alkali treated serum B) may have formed additional complexes of higher dissociability only which were unable to interfere with precipitation of antigen by subsequently added precipitating antiserum. This may be a reason for the better agreement of the amounts of antigen added (column 1) and the amounts of antigen detected (column 8) in the untreated serum B.

Table IV describes the bands of precipitation occurring upon diffusion of CBPA into agar tubes containing the original non-depleted serum, sera A and B, untreated and alkali-treated. The non-depleted, untreated serum gave one band of precipitation consistent with a reaction of antibody with a single component antigen. The untreated serum depleted of precipitating antibody (serum A), failed to reveal a band of precipitation. However, when this serum was treated with alkali and antibody-antigen complex allowed to precipitate, mixtures of the resulting supernate and agar revealed a band of precipitation upon diffusion of CBPA. This band had a similar mobility as the band obtained with the original non-depleted, untreated serum. When the non-depleted serum was treated with alkali and the antibody-antigen complex allowed to precipitate, the supernate, containing both precipitating antibody and the factor precipitating with antigen after alkali treatment, showed only one band in gel diffusion. These data indicate that the material capable of precipitation with antigen only after alkali treatment and the original precipitating antibody probably react with the same component of the antigen solution.

Similar results were obtained with serum B from which both precipitating and coprecipitating antibody were removed. This serum formed two bands

with CBPA. The supernate of a mixture of normal serum with the antigen and antiserum[4] used for removal of coprecipitating antibody gave 2 bands, whether alkali-treated or not. The 2 bands observed in the untreated serum B had a similar mobility as these 2 bands. These bands appear to be derived, therefore, from the antiserum-antigen mixture used for absorption of coprecipitating antibody. When serum B was treated with alkali and antibody-antigen complex allowed to precipitate 3 bands were observed upon diffusion of CBPA. Two of these bands correspond to those of the absorption mixture but the third band had a mobility which is in fair agreement with that of the band of the original non-depleted serum. Again a precipitin-like material which reacted with the same antigenic determinant as the original precipitating antibody was apparently brought out by alkali treatment of the serum portion depleted of precipitating and coprecipitating antibody.

Gel diffusion was also employed to detect any antibody remaining in solution after separation of the precipitates that formed in the alkali-treated and untreated serum A upon addition of the amounts of antigen listed in Table V, column 1. When 4 μg. CBPA nitrogen was added to the treated serum A the supernate was free of antigen (Table V, column 7), and still contained antibody (column 11). When 7 μg antigen were added the precipitating material revealed by alkali treatment was in equivalence, the supernate showing neither antigen (column 7) nor antibody (column 11). With larger doses of antigen the reaction was in antigen excess.

The preceding analysis is consistent with an antiserum reacting with a single component antigen.

Table III shows that depletion of precipitating antibody from the original serum by addition of antigen in the preparation of serum A increased the amount of antibody-antigen complex precipitated after alkali treatment. Removal of coprecipitating antibody with antiserum and antigen in the preparation of serum B further increased the antibody-antigen complex. Apparently some of the antigen added combined with a soluble factor in the serum and increased the amount of antibody-antigen complex precipitated after alkali treatment. The increase of complex precipitated from serum B as compared to serum A is paralleled by a decrease in the maximum amount of material precipitable from the resulting supernates upon subsequent addition of antigen (Table V, columns 3 and 5). The total amount of material precipitable by antigen and disclosed by alkali treatment is reflected by the sum of the following two entities: (a) the difference between antibody-antigen complex nitrogen precipitated from the alkali treated serum depleted of precipitating antibody and the treated non-depleted serum; (b) the maximum amount of nitrogen precipitable upon the addition of antigen to the supernate obtained after removal of the antibody-antigen complex from the alkali-treated serum de-

[4] This antiserum alone produced with the aid of Freund's adjuvants gave 3 bands.

pleted of precipitating antibody. In the case of serum A this is (169–120) + 349 = 398 μg. nitrogen per ml. and in the case of serum B, (253–120) + 228 = 361 μg. nitrogen per ml. This analysis is only qualitative since the amount of antigen incorporated in each of these precipitates is not accurately known. The loss of 37 μg. nitrogen per ml. of material precipitable by antigen from the alkali-treated serum B as compared to A may be due to removal of coprecipitating antibody in serum B (18 μg. nitrogen per ml.) or may reflect experimental error.

The data of this experiment seem to indicate that in sera depleted of precipitating antibody, there appears upon alkali treatment a material which precipitates with specific antigen but not with non-specific protein. Untreated depleted serum fails to form significant precipitates with specific antigen. The precipitates remove antigen from solution. The data suggest, therefore, that alkali treatment converts a non-precipitating factor to a precipitin-like material.

Experiment 3. Conversion of a Non-Precipitating Factor to a Precipitin-Like Material in an Antiserum to an Impure Antigen.—In the preceding experiment a non-precipitating factor combining wih antigen was demonstrated by first depleting antiserum of precipitating antibody, then treating the serum by alkali and allowing antibody-antigen complex to separate, and finally adding antigen to the supernate and estimating the amount of precipitate obtained. For this purpose it was necessary to remove the precipitating antibody completely before alkali treatment. If precipitating antibody would have been left in solution it would have been possible for such antibody to aggregate with non-specific material upon treatment, and this might have given a false increase in the amount of precipitate. Complete removal of precipitating antibody without giving rise to conditions of antigen excess is possible only in an antiserum to a pure antigen composed of a single reactive component. If an antiserum contains antibody to trace impurities of an antigen a large excess of antigen, as shown by Vaughan and Kabat (12), may be necessary to remove all the precipitating antibody. In an antiserum reacting with several components of an impure antigen demonstration of non-precipitating antibody has to depend, therefore, upon the increased antigen removal capacity of the serum after alkali treatment as compared to this property before treatment.

A pool of serum obtained after intravenous injections of BPγG was found to react with 4 components of antigen. The serum was divided into two portions, A and B. Serum A was again divided into two portions. The first portion of serum A was reacted with varying quantities of BPγG labelled with I[131]. The other portion of serum A was treated by alkali, antibody-antigen complex was allowed to separate, and the supernate was reacted with similar quantities of BPγG labelled with I[131]. The radioactivity precipitated from both the treated and untreated sera was compared and non-precipitating antibody was ex-

pressed by any increase in radioactivity precipitated from the treated serum as compared with untreated serum.

To serum B a small amount of antigen was added to deplete it of part of its precipitating antibody. Alkali-treated and untreated portions of serum B (after removal of antibody-antigen complex from the treated portion) were then reacted with radioiodine labelled BPγG as in the case of serum A.

Serum from 4 rabbits was obtained on the 14th day after a single intravenous injection and also 14 days after a second biweekly intravenous injection of BPγG in the amount of 0.1 mg./kg. body weight. The pooled serum was found to precipitate 4 bands in gel diffusion

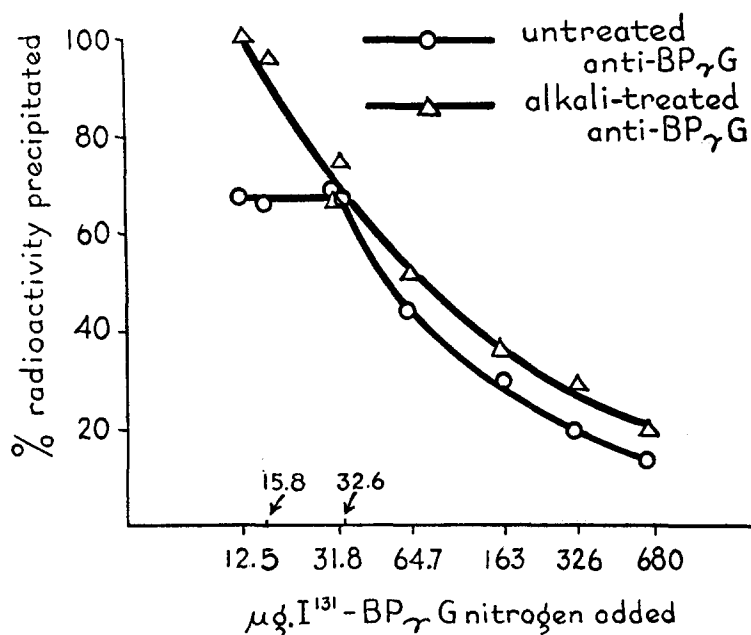Fig. 3. Precipitation of radioiodinated BPγG by alkali-treated and untreated anti-BPγG.

tests with 4 per cent BPγG. The serum was divided into two portions, A and B. Serum A was inactivated at 56° for 30 minutes. Serum B was mixed with 9.7 μg. BPγG nitrogen per ml. of serum (this amount of antigen removed only a fraction of the total precipitating antibody and did not leave antigen in the supernate). The mixture was incubated at 37°C. for 2 hours, left at 1°C. for 11 days and the supernate was obtained after centrifugation at 1000 g at 1°C. for 40 minutes.

Both serum A and the supernate from serum B were divided into two portions. One of each 2 portions was diluted to 4 volumes with respect to the original serum. The other portions of both sera A and B were treated by alkali (resulting in a 4-fold dilution with respect to the original serum) and left to precipitate. Following centrifugation of these four serum portions, 4 ml. samples of each of the resulting supernates were added in duplicates to 1.0 ml. portions of merthiolated saline containing varying amounts of radioiodinated BPγG. The mixtures were left at 1°C. for 11 days, centrifuged at 1000 g at 1°C. for 20 minutes, the precipitates

were washed 3 times with 6.0 ml. portions of 0.16 N sodium chloride solution at 1°C., and their radioactivity was determined. Alkali-treated anti-alternaria serum and purified *Alternaria* antigen (15) were also mixed with radioiodinated BPγG in order to obtain data on non-specific absorption of the radioiodinated antigen.

Fig. 3 shows that more radioactivity was removed by the alkali treated than the untreated serum A, indicating that the treatment disclosed a material which precipitated antigen. The untreated serum precipitated only 68 per cent of 12.5 μg. antigen nitrogen added, while treated serum gave complete precipitation. The supernate from the untreated serum to which 12.5 μg. antigen nitro-
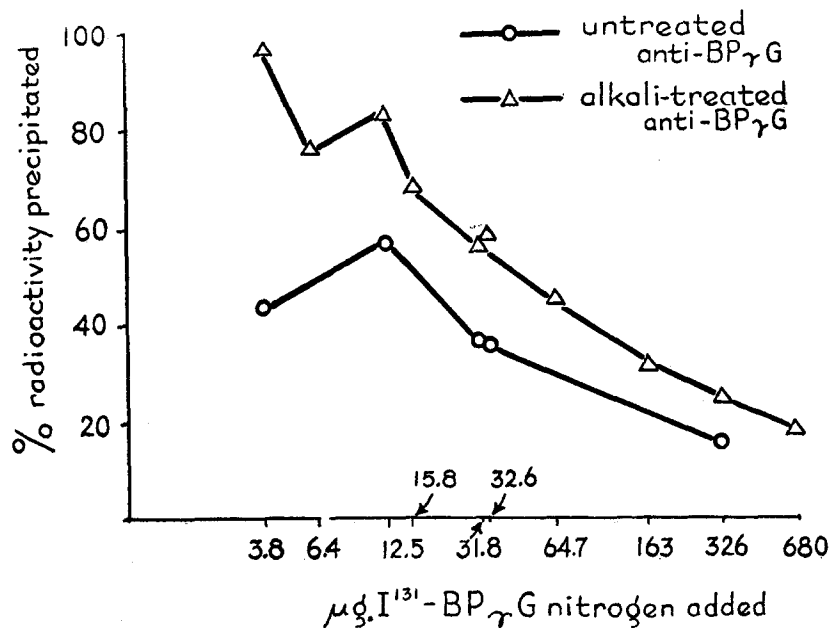

FIG. 4. Precipitation of radioiodinated BPγG by alkali-treated and untreated anti-BPγG depleted of part of its precipitating antibody.

gen was added was free of antigen precipitable with specific antiserum. When increasing amounts of antigen were added, a lesser proportion of the antigen was precipitated by the treated serum. With any amount of antigen except 31.8 μg. nitrogen, more antigen was precipitated by the treated than by the untreated serum.

When the serum was depleted of a part of its precipitating antibodies (serum B), the difference in the proportion of antigen removed from the alkali-treated and untreated serum became accentuated (Fig. 4). This indicates that the depletion removed precipitating antibody from the system without proportionally diminishing the antigen removal capacity of the factor disclosed by alkali treatment.

No significant amount of radioactivity was precipitated by alkali-treated anti-*Alternaria* serum, purified *Alternaria* antigen and radioiodinated BPγG, indicating that non-specific absorption of antigen did not occur.

The experiment demonstrates that there exists in the alkali-treated serum a material which precipitates antigen specifically and which is not disclosed in the untreated serum. This suggests that a non-precipitating serum factor apparently is converted to a precipitin-like material, that this can be accomplished in the presence of precipitating antibody and that it holds true for sera reacting with multiple components of antigen as well as for single component antibody-antigen systems.

Experiment 4. Increase of Non-Precipitating Antibody-Antigen Complex upon Addition of Antigen to Antiserum to Powdered (Non-Crystalline) BPA and Freund's Adjuvants Depleted of Part of Its Precipitating Antibody.—The antisera used for the preceding experiments were produced by infrequent intravenous injections of relatively large quantities of protein. With this method appreciable amounts of material precipitable with antigen after alkali treatment are produced but no excessive quantities of precipitating antibody form. The present experiment was undertaken to learn whether formation of large amounts of precipitating antibody such as occurs in immunization with Freund's complete adjuvants (2, 3) parallels or precludes the appearance of material precipitable with antigen after alkali treatment.

By serial additions of small amounts of antigen, antiserum to powdered BPA was depleted of most of its precipitating antibody. This comprised all the antibody to one of the 3 antigen components of CBPA with which the serum reacted. The partially depleted serum was then mixed with varying additional quantities of the antigen and the resulting precipitates were removed. The ensuing supernates were treated with alkali and antibody-antigen complex was allowed to separate. The amounts of antibody-antigen complex precipitated after treatment by these supernates were compared with the complex precipitated by depleted serum to which no additional antigen was added. The solutions remaining after separation of antibody-antigen complex from the alkali treated supernates and untreated controls were tested for excess antigen and antibody. The number of antigen components capable of reacting with these solutions was inferred from gel diffusion tubes.

A pool of serum from rabbits immunized with powdered BPA and Freund's adjuvants was obtained. Upon diffusion of 4 per cent CBPA into serum agar mixtures 3 bands resulted. Apparently there are small amounts of impurities in the CBPA that can be detected if a less purified antigen is used for immunization, particularly when in Freund's complete adjuvants (2, 3).

The serum was depleted of 712 μg. precipitating antibody nitrogen per ml. by four successive additions each of 13 μg. CBPA nitrogen per ml. of serum. After each of the first three additions the serum was incubated at 37°C. for 30 minutes and centrifuged at 1000 g at 1°C. for 20 minutes. After the fourth addition it was left at 37°C. for 2 hours and at 1°C. for 7 days

and centrifuged at 1000 g at 1°C. for 40 minutes. As a result of this procedure the serum was free of antibody in qualitative tests with 4 μg. but not with 40 μg. CBPA nitrogen per ml. undiluted serum, indicating that apparently an antibody to a trace impurity of CBPA was still left in the serum (12). Tests for free antigen were negative.

Portions of depleted serum were mixed with different quantities of CBPA or with merthiolated saline. They were incubated at 37° for 2 hours, left at 1°C. for 7 days, and centrifuged at 1000 g at 1° for 20 minutes. Table VI, column 2, gives the nitrogen determinations of the washed precipitates representing residual precipitating antibody in the depleted serum. Each of the resulting supernates was divided into two portions. One of these portions was treated

TABLE VI

Precipitates Formed in Alkali-Treated Supernates from Mixtures of Varying Amounts of CBPA with Antiserum to Powdered BPA and Freund's Adjuvants Depleted of Part of Its Precipitating Antibody

(1) μg. CPBA added per 1.2 ml. depleted serum (1.0 ml. un- diluted serum)	(2) μg. N precipi- tated per ml. undiluted serum	(3) μg. N per ml. undiluted serum spontane- ously pre- cipitated from untreated super- nates of 2	(4) Ex- cess anti- gen	(5) Bands precipitated by diffusion of 4 per cent CPBA through a mixture of equal parts of supernates and agar	(6) μg. N/ml. undiluted serum spontane- ously pre- cipitated from alkali- treated supernates of 2*	(7) Increase due to addition of antigen in μg. N/ml. undiluted serum spon- taneously precipitated from alkali- treated supernates of 2	(8) Ex- cess anti- gen	(9) Bands precipi- tated by diffusion of 4 per cent CBPA through a mixture of equal parts of super- nates and agar
				Tests on supernates from precipitates in column 3				Tests on supernates from precipitates in column 6
0	0	0	−	0.7 M, 0.6 MH‡	49		−	1.8 L, 0.7 M, 0.6 H‡
2	0	0	+	0.7 M, 0.6 MH	78	29	−	1.6 L, 0.8 M, 0.6 H
4	0	0	+	0.7 M, 0.6 H	112	63	+	2.4 LL, 0.8 MH, 0.6 H
11	11	13	+	0.7 MH, 0.6 H	143	94	+	0.8 MH, 0.6 H
21	17	Trace	+	0.7 MH, 0.5 H	117	68	+	0.8 MH, 0.6 H
43	11	11	+	0.7 MH, 0.5 H	122	73	+	0.8 MH, 0.6 H
86	10	Trace	+	0.8 MH, 0.6 H	189	140	+	0.8 MH, 0.6 H
108	19	0	+	0.8 MH, 0.6 H	118	69	+	0.8 MH, 0.6 H
216	Trace	0	+	0.8 MH, 0.6 H	196	147	+	0.8 MH, 0.6 H
432	15	0	+	0.8 MH, 0.6 H	180	131	+	0.8 MH, 0.6 H

* Corrected for spontaneous precipitates, if any, occurring in untreated supernates (column 3).

‡ See footnote of Table IV.

with alkali and made up with merthiolated saline to 4 volumes with regard to the original serum. The other (control) portion was not treated but was diluted similarly with merthiolated saline. 4 ml. triplicate samples of each supernate were left at 1°C. for 11 days. The tubes were then centrifuged at 1000 g at 1°C. for 20 minutes, a set of new supernates was collected and the nitrogen content of the washed precipitates determined. The new supernates were tested by gel diffusion for excess of material reacting with antigen. They were also examined for excess antigen by addition of equal volumes of anti-CBPA produced with Freund's adjuvants. The tubes were read after incubation at 37° for 1 hour and storage at 1°C. for 1 day.

Table VI, column 2 gives the amounts of precipitate obtained by the addition of varying quantities of antigen to serum depleted of most of its precipitating antibody. Column 3 shows that most of the supernates from these precipitates

when simply diluted and stored at 1°C. for 11 days, but not treated with alkali, failed to form additional precipitates. The small amount of precipitate observed in some of the samples apparently was due to incomplete precipitation of precipitating antibody after addition of antigen to the partially depleted serum. However, when the supernates were treated with alkali large amounts of precipitate formed (column 6). The amount of these precipitates exceeded the amount of antibody-antigen complex in the depleted serum, *i.e.* the amount of precipitate obtained from the alkali-treated sample of depleted serum to which, instead of antigen, merthiolated saline was added. This increase is shown in column 7. These results are consistent with the assumption that a soluble complex was formed by addition of antigen to the serum depleted of most of its precipitating antibody. This complex apparently precipitated upon alkali treatment and resulted in an increase of antibody-antigen complex when compared with the amount of complex precipitated from the depleted serum to which no antigen was added. No regular pattern was observed, however, in this increase of complex when increasing amounts of antigen were used. This may be due to the presence of multiple antibody-antigen systems in the serum. Such irregular increase of precipitation from alkali-treated serum with increasing amounts of antigen was not observed in sera obtained after intravenous injections of CBPA that reacted with a single antigen component only.

Tests on the supernates remaining after separation of antibody-antigen complex from the alkali-treated samples are given in columns 8 and 9 and tests on the corresponding untreated controls in columns in columns 4 and 5. The supernate obtained after alkali treatment from depleted serum admixed with 2 μg. CBPA nitrogen per ml. original serum was free of antigen (column 8). Antigen was left in the supernate of the corresponding untreated sample (column 4). Hence the precipitate obtained after treatment incorporated antigen. (The alkali treatment itself does not destroy the antigen in mixtures of CBPA and normal serum.) Gel diffusion revealed 2 bands in the supernates from the untreated samples (column 5) (the original unabsorbed serum had 3 bands). These 2 bands did not disappear from the supernates with any of the amounts of antigen used, showing that they are due to a trace impurity of the antigen too small to neutralize all the antibody with any of these antigen doses (12). Two bands of the same mobility appeared in the supernates from the alkali-treated samples (column 9). However, a third band was also apparent in the supernate from the sample to which no antigen was added and in the supernates from the samples admixed with up to 4 μg. antigen nitrogen per ml. original serum. Since the original serum precipitated 3 bands, and the depleted untreated serum 2 bands, reappearance of a third band after alkali treatment of the depleted serum is consistent with the existence in the latter of a material reacting with the main component of antigen without forming precipitates but precipitating with antigen after alkali treatment. In the sample admixed

with 4 μg. antigen nitrogen per ml. of original serum, the supernate obtained after alkali treatment contained both antigen and free antibody, reacting with each of the 3 antigen components. This may indicate a possible coexistence of antigen and the material disclosed by alkali treatment.

GENERAL DISCUSSION

Antisera to which no antigen is added usually form precipitates upon alkali treatment. These precipitates are derived from circulating non-precipitating antibody-antigen complex (1). Experiments 1 and 4 of the present work show that in antisera depleted of precipitating antibody the addition of antigen increases the amount of these precipitates obtained after alkali treatment. This increase may be due to one of two causes. Either the antibody-antigen complex contained in the serum is by itself capable of binding antigen beyond the amount it already contains, or there may be present in the serum another soluble factor which precipitates with antigen after alkali treatment. In Experiments 2 and 3 antibody-antigen complex was allowed first to separate from alkali-treated sera which had been depleted in varying degrees of precipitating antibody, and antigen was added only later. This resulted in further precipitation, while untreated sera did not produce precipitates. The material in alkali-treated serum precipitable with antigen is, therefore, distinct from the antibody-antigen complex originally present. It fails to precipitate from untreated serum. After treatment it resembles precipitating antibody by its capacity to precipitate specifically with antigen but not with saline nor with non-specific protein, and by its capacity to bind antigen during this precipitation. Gel diffusion tests showed that the material revealed by alkali treatment of serum which had been depleted of precipitating antibody reacted with the same component of antigen as the precipitating antibody of non-depleted, untreated serum. These findings support the conclusion that the alkali treatment converts a non-precipitable factor to a precipitin-like material.

This precipitin-like material differs from precipitating antibody in that maximal precipitation occurs in the antigen excess zone and that apparently higher concentrations of antigen are needed for inhibition. In the region of moderate antigen excess close to the equivalence zone considerable amounts of the material may apparently coexist with antigen in the supernates. This may be due to a greater dissociability of the antigen from the material disclosed by alkali treatment than from precipitating antibody in untreated serum and resembles the behavior of precipitating antibody with a cross-reacting antigen such as described by Pennel and Huddelson (16).

The nature of the alteration that causes appearance of precipitin-like material on alkali treatment is not understood. Of course, without change in structure the precipitin like material would never form. The precipitates obtained reflect, therefore, merely a means of estimating the presence of such material. They

do not represent the unaltered non-precipitating material that may have been originally found in the serum. Their behavior after dissociation does not permit definite conclusions about the nature of their mother substance in the native serum. However, the cause of precipitation is not denaturation. If immune sera are treated under conditions favoring denaturation, such as pH 12.6 for 6 minutes at room temperature, no precipitates form upon neutralization and storage at 1°C. showing that denaturation destroys rather than produces the capacity of forming precipitates.

Aggregation of precipitating antibody with non-specific material such as occurs in treatment of serum with guanidine (17) or heat (18) could be a factor in increasing the amount of precipitate obtained with antigen after alkali treatment. In order to exclude this possibility, care was taken in the present experiments to deplete precipitating antibody from serum before treatment. After depletion no significant precipitation occurred with antigen in untreated sera reacting with a single component of antigen. Any precipitating antibody that could nevertheless have remained in solution in untreated depleted serum depends on the solubility of antibody-antigen precipitates, which is 2 μg. nitrogen per ml. as determined by the effect of volume on precipitation at 1°C. for 11 days (19). Since 5 ml. volumes (4 ml. of diluted serum and 1 ml. of antigen) were employed for each precipitation, not more than 10 μg. nitrogen of precipitating antibody in combination with antigen could have remained in solution in equivalence or antibody excess of untreated serum. After alkali treatment up to 306 μg. nitrogen were precipitated specifically from serum mixed before treatment with antigen in antibody excess. If aggregation would have been responsible for this phenomenon, antibody would have aggregated with 30 times its weight of protein. It is unlikely that specificity would have been retained under conditions of so extensive aggregation since it is known that extensive aggregation of antibody in the presence of serum proteins abolishes or decreases its specific precipitability (17, 18). Also, serum devoid of non-precipitating specific factors but containing traces of precipitating antibody and antigen retained in solution by nature of their solubility remains clear after alkali treatment, just as normal serum does (19). The fact that immune sera, depleted of precipitating antibody by the addition of antigen, show an increase of antibody-antigen complex precipitating after dissociation must, therefore, be attributed to other causes than extensive aggregation of any precipitating antibody and antigen that remained in solution after depletion.

Furthermore, in some experiments antiserum was depleted of precipitating antibody, treated with alkali, and antibody-antigen complex was allowed to precipitate. The remaining supernates contained material precipitating specifically with antigen. If alkali treatment had produced precipiation of a residue of traces of precipitating antibody and antigen which had remained in solution in the depleted serum, this material would have separated along with antibody-

antigen complex, and not upon the subsequent addition of antigen to the resulting supernates.

Finally unpublished experiments have shown that isolated precipitating antibody will aggregate with 76 to 150 per cent its weight when treated with alkali in the presence of normal serum, lesser aggregation occurring in antibody excess than in antigen excess. The amount of antibody-antigen complex precipitating after treatment of sera which had been depleted of precipitating antibody in the present experiments exceeded by far the amount expected if the complex were derived from any traces of precipitating antibody and antigen that could have remained in solution in untreated serum. When precipitating antibody and radioiodinated antigen in antigen excess and in equivalence are treated with alkali in the presence of normal serum, neutralized, and allowed to reprecipitate, the same amount of radioiodine-labelled antigen will be lost in the combined supernates and washings of treated as of untreated precipitates. The increased amounts of antigen precipitated by treated as compared to untreated sera in the present experiments are, therefore, not due to impairment of solubility of any precipitates derived from precipitating antibody and antigen, but result from the effect of alkali treatment on a factor other than precipitating antibody.

The alkali treatment revealed material precipitable with antigen in sera depleted of precipitating antibody by single additions of antigen in antigen excess (Experiment 1) or by repeated additions of antigen in antibody excess (Experiments 2 and 4). It does not depend, therefore, on the ratio of the antigen employed and the precipitating antibody removed during depletion of the serum. This is further indication that the material disclosed by alkali treatment is not derived from solubilization of any precipitates of precipitating antibody and antigen.

All these considerations support the conclusion that the alkali treatment discloses a material precipitating with antigen which before treatment has been in the state of a non-precipitating entity. The precipitates obtained after treatment are not derived from any residual precipitating antibody retained in solution before treatment.

What was the original nature of the precipitin-like material before the interference of the alkali treatment? Does the treatment complete the production of a precipitating antibody from a percursor or does it convert a non-precipitating antibody of serum to a precipitin-like material? When precipitating antibody is removed from serum by small serial additions of antigen in antibody excess and the depleted serum is treated with alkali, the amount of antibody-antigen complex precipitated exceeds that of the original serum (Table III). Apparently some of the antigen added is bound by a factor in the untreated serum and prevented from precipitation. This would indicate that the material disclosed by alkali treatment is derived from a non-precipitating antibody preformed in the untreated serum. It is not synthesized from an inactive precursor during

the treatment. It is of interest that none of the antigen seemingly bound by non-precipitating antibody during absorption of precipitating antibody by small amounts of antigen can be detected by addition of precipitating antibody. Apparently the dissociability of some of the complexes between non-precipitating antibody and antigen formed in untreated serum is so low that sufficient free antigen is not available for precipitation with precipitating antibody. A more direct demonstration of strong binding of antigen by a non-precipitating factor in serum has recently been obtained by Robert Feinberg (14). This is also suggested from some of the data of Fig. 3. Apparently it is this strong antigen binding factor which is, possibly along with other soluble factors of lesser binding strength, converted by the alkali treatment to precipitin-like material. It appears feasible that upon addition of small amounts of antigen to antiserum complexes of low dissociability form first, while other complexes of higher dissociability are only revealed when an excess of antigen is used. Considerable dissociation of some complexes under conditions of antigen excess has already been indicated in the preceding discussion by the coexistence of antigen and antibody in some of the supernates obtained from alkali-treated serum and excess antigen.

Among the possibilities for the mechanism by which the alkali treatment converts non-precipitating antibody to precipitin-like material, perhaps the following two may deserve consideration.

It is feasible that some antibody is non-precipitating because it is complexed with a non-specific inhibitor that keeps it in solution. It is known that the alkali treatment effects dissociation of various complexes, such as precipitating antibody and antigen (20), complexes of complement with antibody and antigen (1) and thrombin-antithrombin complex (21). Perhaps the treatment results in a similar separation of non-precipitating antibody from a hypothetical inhibitor. It may even cause destruction of such an inhibitor, just as it effects destruction of complement concurrent with dissociation of antibody-antigen-complement complex.

Another possibility may be a masked combining site of non-precipitating antibody. Perhaps the alkali treatment permits just that degree of unfolding necessary to expose such a combining site.

The data of this paper neither suggest nor preclude a similarity of the non-precipitating antibody described by Heidelberger and coworkers (13) (coprecipitating antibody) and at least some of the non-precipitating antibodies disclosed by alkali treatment. Because of the large amounts of non-precipitating antibody remaining after removal of relatively small quantities of coprecipitating antibody (tested for completeness of absorption), it is not possible to ascertain whether this removal decreased the total amount of non-precipitating antibody revealed by alkali treatment.

The formation of non-precipitating antibody in immunization explains the regular appearance of large amounts of circulating antibody-antigen complex.

Apparently during immunization most of the antigen is removed by precipitating antibody and metabolized subsequently. Yet some of the antigen or material reacting like antigen may persist in circulation in combination with non-precipitating antibody and form soluble antibody-antigen complex (1).

SUMMARY

Brief exposure of serum to alkali in the cold seems to convert non-precipitating antibody to a precipitin-like material. This was disclosed by the following lines of evidence.

1. Immune sera, after addition of antigen and removal of the ensuing precipitates, yielded after alkali treatment more antibody-antigen complex than did sera to which no antigen was added.

2. When immune sera were depleted of precipitating antibody, treated by alkali, and antibody-antigen complex was allowed to precipitate, the resulting supernates formed additional precipitates upon admixture of specific antigen. No precipitation occurred upon addition of non-specific protein to the treated supernates or the addition of specific antigen to depleted sera not treated by alkali.

3. Alkali-treated immune sera precipitated more antigen than untreated sera.

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