THE ESTIMATION OF CIRCULATING ANTIBODY-ANTIGEN COMPLEX*

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It has generally been observed that during the course of immunization the appearance of antibody in circulation follows the disappearance of antigen (1, 2). Appreciable amounts of free antigen and its homologous free antibody apparently do not coexist in a single system (3). Existence of complexes consisting of circulating bound antibody and antigen could depend upon several factors. The earliest antibody formed during immunization may conceivably combine with antigen present in circulation. Even if the aggregates formed fail to precipitate they may escape detection by the serologic methods thus far developed. Present immunologic methods adequately detect free antibody by reactions resulting from addition of antigen, and similarly, free antigen can be detected by addition of antibody. These methods, however, would not identify antibody-antigen complex combined *in vivo*.

It seemed worth while to learn whether bound antibody and antigen may coexist in immune serum without showing serologic activity as such, and whether any latent serologic activity of such a complex can be detected by dissociation and reassociation *in vitro*. Previous studies suggested possible approaches. Thrombin formed in circulation is progressively inactivated by combination with antithrombic substances to form inert thrombin-antithrombin complexes. Active thrombin can be recovered by dissociating thrombin-antithrombin complex by brief alkali treatment in the cold. Addition of ethanol to the dissociated constituents prevents reassociation upon subsequent neutralization (4). A similar procedure has also been employed in the dissociation of precipitated complexes of antigen and antibody. The antigen used was coupled with acidic groups capable of reacting with alumina at an alkaline pH to form insoluble precipitates (5). This allowed recovery of dissociated antibody that was 88 to 100 per cent pure. The combining ratio of antigen with antibody was not altered. It was found that dissociation began at a pH of approximately 11 and was more pronounced the higher the pH. Electrophoretic, sedimenta-

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tion, and viscosity studies of gamma globulin treated in a similar manner at 2°C. for 15 minutes failed to disclose appreciable irreversible alteration in molecular structure at pH 11.4 although some changes took place at pH 12.6 (6). Kleinschmidt and Boyer have reported marked changes in gamma globulin exposed to alkali at the same temperature for from 2 to 24 hours (7).

Since brief treatment with alkali at low temperature seemed to effect dissociation of both thrombin-antithrombin and precipitated antigen-antibody complexes, sera from immunized rabbits were similarly treated and the serologic patterns determined.

Methods

Male rabbits weighing 3 to 4 kg. were given a single intravenous injection of beef plasma albumin or beef plasma globulin (Armour) in a dose of 1 gm. per kg. body weight. Other rabbits were injected intramuscularly with 80 mg. of the same antigens together with adjuvants by the method of Freund (8). Blood was obtained before injection and at varying intervals thereafter. The sera were quick frozen at about −60°C. and stored at about −23°C. until used.

After thawing, the sera were titrated for complement activity (9) and then inactivated for 30 minutes at 56°C. The precipitable antigen and antibody content of portions of the otherwise untreated sera were estimated by quantitative precipitation analysis (10–12). Other suitably diluted portions of these sera were cooled to 1°C., adjusted with NaOH to a pH of 12.6 for a period of 6 minutes, then readjusted with HCl to the original pH of the serum. This constituted the dissociation procedure. Portions of the processed sera, together with untreated individual control specimens, were incubated at 37°C. for 2 hours followed by 4 days at 1°C. Any precipitates that formed were removed by centrifugation at 1°C. and washed with saline. Their protein nitrogen content was estimated by the same procedure used to determine free antigen and antibody in undissociated portions of the respective sera. Other portions of the processed sera were treated with an excess of homologous and heterologous antigens in order to secure data pertaining to their specificity.

Determination of Free Complement.—Complement titrations were carried out with freshly thawed sera by the method of Wadsworth, Maltaner, and Maltaner (9). The results were expressed as the number of 50 per cent hemolytic units of complement contained in 1.0 ml. of serum.

Determination of Free Antigen.—Free antigen was determined by the method of Heidelberger and Kendall (11), which involves the addition of an excess of calibrated antiserum to a small amount of antigen. Determinations were made in triplicate or quadruplicate.

Determination of Free Antibody.—The amount of antibody was calculated by the method of Heidelberger and Kendall (10) from nitrogen determinations in triplicate or quadruplicate of the precipitates obtained in the equivalence zone. Sera containing amounts of antibody per milliliter that were insufficient to precipitate 4 μg. antigen nitrogen completely gave definite turbidites with antigen but the quantity of precipitate was considered too small for quantitative estimation and was reported therefore as "trace."

Dissociation and Reassociation of Sera.—The inactivated sera were chilled and 1 volume of serum (usually 5 to 9 ml.) was mixed with an equal volume of chilled 0.16 N sodium chloride solution containing merthiolate 1:10,000 (sodium ethyl mercurithiosalicylate). The pH of the mixture was determined at 1°C. It varied between 8.4 and 9.1 in different samples. The mixture was then brought to pH 12.6 (1°C.) with 0.1 N and 0.01 N sodium hydroxide solution. (The sodium hydroxide solutions had been adjusted to 0.16 N Na^+ with sodium chloride.) The mixture was held at this pH and temperature for 6 minutes and neutralized to its original pH with 0.1 N and 0.01 N hydrochloric acid. (The hydrochloric acid solutions had been ad-

justed to 0.16 N Cl^- with sodium chloride.) The neutralized mixture was then made up to 4 volumes (usually 20 to 36 ml.) with 0.16 N sodium chloride solution containing merthiolate 1:10,000. Similar pH adjustments at the required temperature have been reported previously for a different procedure (13) and details are described there. Control samples of undissociated sera were diluted with 3 volumes of 0.16 N sodium chloride solution containing merthiolate 1:10,000.

Estimation of Precipitates in Dissociated and Reassociated Sera.—5 to 7 ml. of the diluted alkali-treated sera or diluted untreated controls were incubated at 37°C. for 2 hours and left at 1°C. for 4 days. The tubes were then centrifuged at 1000 *g.* at 1°C. for 40 minutes. The supernates were decanted and observed for 3 more days at 1°C. The precipitates were washed and centrifuged twice with 8.0 ml. portions of 0.16 N sodium chloride solution at 1°C. Their protein content was then determined with the Folin-Ciocalteau reagent by the method of Heidelberger and MacPherson (12). The reagent was standardized by comparison with Kjeldahl nitrogen determinations. Results were expressed in micrograms of nitrogen per milliliter of undiluted serum. Determinations were made in triplicate or quadruplicate. The untreated controls averaged 3.2 μg. nitrogen per ml. (maximum 5, minimum 2); hence 3 μg. nitrogen has been subtracted from the values obtained for each precipitate from dissociated sera. Amounts of precipitate smaller than 10 μg. nitrogen gave definite turbidities but were considered too small for quantitative evaluation. They were therefore reported as "trace."

Specificity of Precipitates.—Samples of whole suspensions were divided into 5 portions. To each portion was added an equal volume of 0.16 N sodium chloride solution or solutions of beef plasma albumin or globulin made by dissolving 1 gm. in 7.5 ml. or in 50 ml. of 0.16 N sodium chloride solution. This test was done as soon as possible after dissociation and reassociation of the sera. When the dissociated sera contained much free antibody, washed precipitates were used in place of whole suspensions. The tubes were incubated at 37°C. for 10 minutes to 2 hours, sometimes followed by storage at 1°C. for 12 hours. Dissolution of precipitates with specific antigen but not with non-specific protein or sodium chloride solution was considered to indicate their specificity.

Complement Fixation by Dissociated and Reassociated Sera.—The method was adapted from that of Wadsworth, Maltaner, and Maltaner for quantitative complement fixation (14). To 0.2 ml. portions of dissociated and reassociated or control serum, were added 2, 3, 6, 9, or 12 units of guinea pig complement in 0.1 ml., followed by 0.1 ml. of 0.16 N sodium chloride solution. The tubes were incubated for 30 minutes at 37°C. Then 0.2 ml. of a 2.5 per cent suspension of sensitized sheep erythrocytes was added and the tubes were incubated at 37°C. for 15 minutes. The amount of complement fixed was expressed in terms of 50 per cent hemolytic units (9) using conversion factors derived by the method of Wadsworth, Maltaner, and Maltaner (14). The reported values were calculated by subtracting the number of units of complement fixed by the undissociated control serum from that obtained for the dissociated and reassociated serum. The results were considered qualitative since information concerning the proportion of antibody and antigen in the complex was lacking.

EXPERIMENTAL RESULTS

Immune Response of Rabbits after Intravenous Injection of a Single Large Dose of Beef Plasma Globulin

6 rabbits were given a single injection of beef plasma globulin in a dose of 1 gm. per kg. body weight. The succeeding changes in the free antigen, antibody, and complement content of undissociated sera were followed. This pattern was compared with corresponding fluctuations in the amount of precipitate developed and complement fixed by the same sera after dissociation.

The data shown in Fig. 1, obtained from study of 1 rabbit, represent the results with all 6. Free antigen rapidly diminished; it was no longer found after the 10th day. The first appearance of serologically detectable antibody was on the 12th day. Only a trace of free antibody too small for nitrogen analysis was detected. It persisted up to the 33rd day. In serum taken on the 7th day, when a large amount of free antigen was still present, precipitates

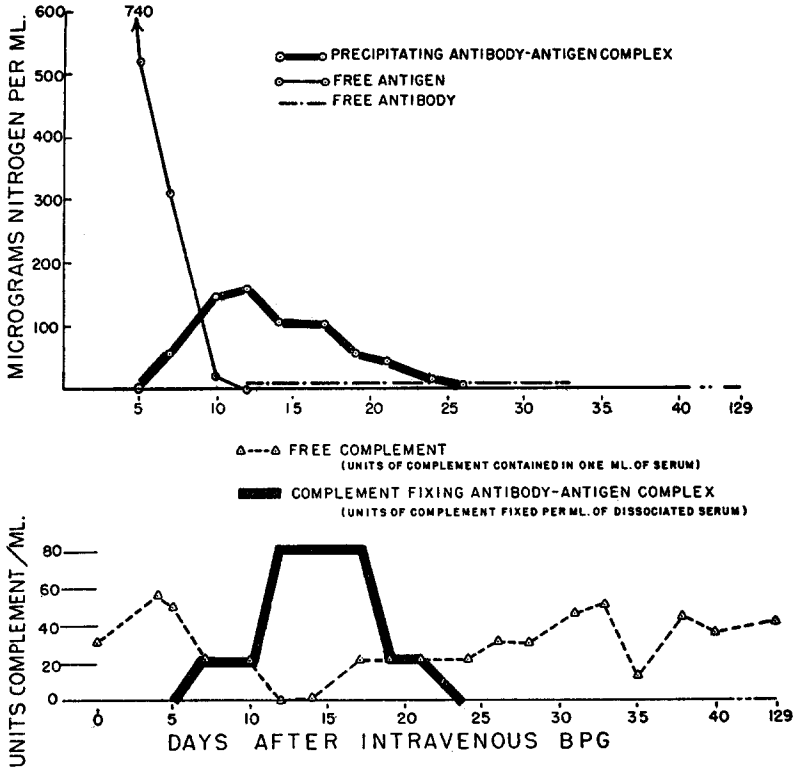

FIG. 1. Immune response after intravenous injection of a single large dose of beef plasma globulin.

appeared spontaneously after the dissociation treatment without the addition of antigen or antibody. The maximum amount of such precipitation was found on the 12th day, when antigen was no longer detected. None was noted after the 24th day, although a trace of free antibody was still present. These precipitates were soluble in an excess of beef plasma globulin but not beef plasma albumin. The complement activity of the fresh sera was completely inhibited at a time when spontaneous precipitation was at a maximum. The capacity of the alkali-treated sera to fix complement paralleled closely the level of the spontaneous precipitates obtained after dissociation.

Immune Response of Rabbits after Intravenous Injection of a Single Large Dose of Beef Plasma Albumin

The experiment done with beef plasma globulin was repeated with beef plasma albumin. A single intravenous injection of 1 gm. per kg. body weight was given to 4 rabbits. The data obtained with 1 rabbit, presented in Fig. 2, are typical of the results with all 4.

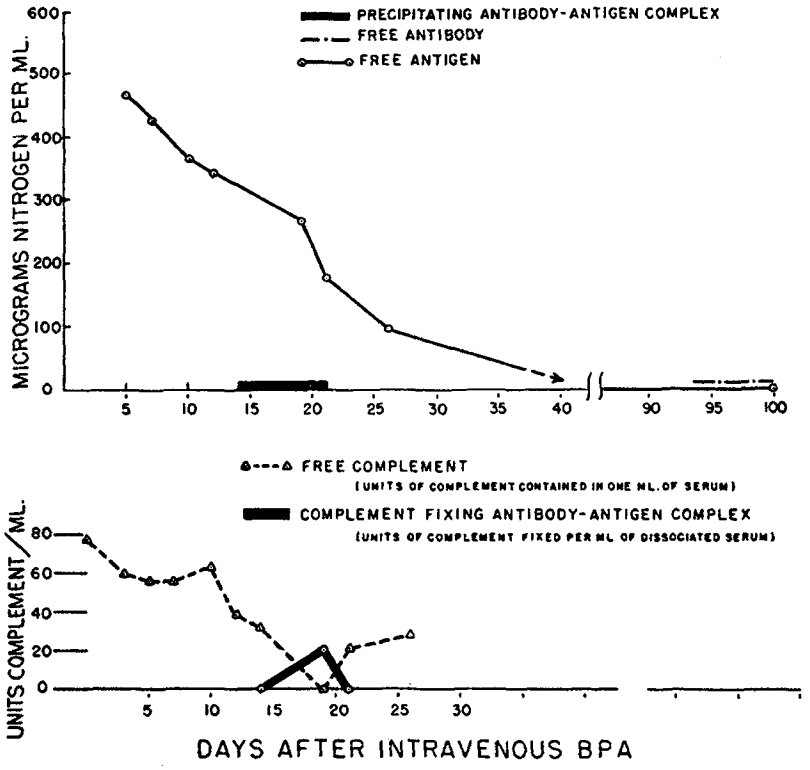

FIG. 2. Immune response after intravenous injection of a single large dose of beef plasma albumin.

Free antigen persisted for a much longer time than it did after intravenous injection of beef plasma globulin. Such persistence of antigen would indicate a poor immune response according to Dixon and coworkers (15, 2). It is in agreement with the findings of Janeway and others (1) that large doses of beef plasma albumin persist in circulation much longer than large doses of beef plasma globulin. No free antibody was found until the 100th day. Traces of spontaneous precipitates appeared in the dissociated sera during the 3rd week after injection and only on the 19th day was there evidence of complement fixation by the dissociated sera. The spontaneous precipitates were soluble in an excess of beef plasma albumin but not in beef plasma globulin. The complement activity of the fresh sera was completely inhibited at the time of ap-

pearance of precipitation and fixation of complement in the dissociated sera, indicating a limited immune response in the 3rd week.

Immune Response of Rabbits after Intramuscular Injection of Beef Plasma Globulin with Adjuvants

The plan of the third experiment was identical with that of the first except for the facts that immunization was carried out with the aid of adjuvants and the amount of antigen was reduced markedly. Each of 2 rabbits was injected intramuscularly with 80 mg. of beef plasma globulin. Similar results were obtained with the 2 rabbits; Fig. 3 shows one set of data.

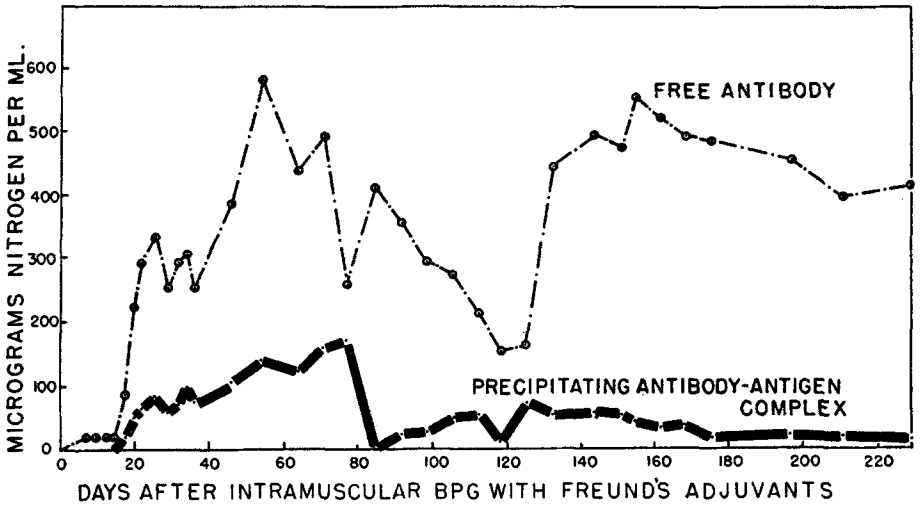

FIG. 3. Immune response after intramuscular injection of beef plasma globulin with Freund's adjuvants.

Tests for free antigen were begun with sera taken on the 8th day. None was found in any of the specimens. Free antibody appeared on the 8th day. Large amounts persisted throughout the period of observation. Spontaneous precipitates in the dissociated sera formed only after free antibody was noted. After the 85th day only small amounts were found at irregular intervals. Those obtained before the 85th day were soluble in an excess of beef plasma globulin but not in beef plasma albumin. Some of the precipitates obtained after the 85th day, however, failed to dissolve in beef plasma globulin or in a mixture of an excess of beef plasma globulin with a 1:5 dilution of lyophilized BCG tuberculin in saline.

The dissociated sera failed to fix complement. Free complement did not show significant changes during the period of observation.

Immune Response of Rabbits after Intramuscular Injection of Beef Plasma Albumin with Adjuvants

The experiment with intramuscular injection of beef plasma globulin with adjuvants was repeated with beef plasma albumin. Each of 7 rabbits was injected intramuscularly with 80

mg. of beef plasma albumin. The data obtained with 1 rabbit, presented in Fig. 4, are typical of the results with all 7.

The response resembled that following intramuscular injection of beef plasma globulin with adjuvants. Again spontaneous precipitates in the dissociated sera did not appear until after the free antibody. The spontaneous precipitates were soluble in an excess of beef plasma albumin but not in beef plasma globulin. Free antigen was only detected on the 8th day, the first specimen examined, and was not seen in other animals of this series. Free antibody persisted in

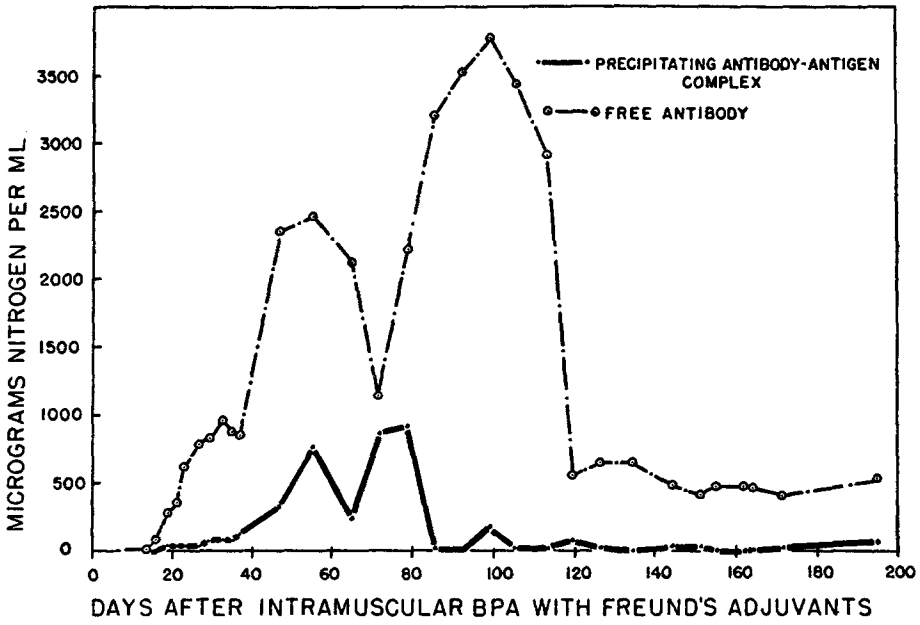

FIG. 4. Immune response after intramuscular injection of beef plasma albumin with Freund's adjuvants.

large amounts throughout the period of observation. The spontaneous precipitates in the dissociated sera reached high levels and dropped only by the 92nd day, following which only small amounts persisted at irregular intervals. The amount of free complement did not show significant changes in this series of observations. The dissociated sera failed to fix complement.

DISCUSSION

Of great concern is the question whether the precipitates observed in sera dissociated by alkali are specific or whether they are non-specific aggregates formed as a result of the technic. A question of lesser importance appears to be whether the precipitates observed—if composed of antibody and antigen—are native or degraded materials. As the precipitates were observed only in im-

mune sera and not in preimmunization sera the phenomenon appears to be linked with immunization. The question should be considered whether there occur any non-specific changes in the sera leading to increased precipitability of protein as a secondary result of immunization. Thus, the development of so called "cryoglobulins" (16, 17) could explain the precipitates developed in the immune sera, since all these were stored at 1°C. for 4 days. None of the untreated immune sera, however, developed a precipitate upon storage. When beef plasma globulin was injected intravenously, the precipitates in the dissociated sera were present only during the late stage of antigen disappearance and during the early stage of antibody formation. In the case of intramuscular immunization with adjuvants, precipitates persisted in reduced but detectable quantities much longer, yet failed to appear in most instances in the late stages of immunization when free antibody was still present in significant amounts. The time of development of the precipitates, their increase and decline in relation to the disappearance of free antigen and appearance of antibody support the tentative conclusion that they represent antigen-antibody complex.

An immune precipitate, in general, should satisfy at least 2 requirements: specificity, and a zone phenomenon. Both these criteria were satisfied by the precipitates observed after alkali treatment and neutralization. They were soluble in a large excess of specific antigen and failed to dissolve in unrelated proteins. This seems to favor the concept that the precipitates obtained were specific but does not exclude the possibility that drastic alterations in the properties of their constituents (antibody and antigen) had occurred as a result of the alkali treatment.

The experiments reported are concerned with antigen and antibody bound *in vivo*. Unpublished studies of the formation and dissociation of similar antigen-antibody products *in vitro* yielded data consonant with the results *in vivo*. Protein-antiprotein precipitates were treated repeatedly with portions of an excess of guinea pig complement until no more complement could be fixed by the suspensions whether they were inactivated at 56°C. for 30 minutes or not. Such precipitates saturated with complement and inactivated were treated with alkali and neutralized in the cold. Control precipitates were neither absorbed with complement nor exposed to alkali. The two sets of precipitates fixed the same quantity of complement. Thus the dissociation procedure removed bound complement and permitted the precipitates to fix more. It cannot be inferred, however, that a similar mechanism necessarily applies to the complement-fixation data with soluble antigen and antibody bound *in vivo*.

Certain other observations are as yet unexplained. Soluble antibody-antigen complexes can be formed *in vitro* that fail to precipitate spontaneously or after addition of free antigen or antibody. Precipitation does take place after alkalization and neutralization in the cold. The precipitates are soluble in excess specific antigen. Further experiments are being done to explain these phenomena.

What might be termed provisionally antibody-antigen complex was apparent after intravenous injection of a single large dose of protein at a time when an excess of antigen was still present in circulation and before an appearance of free antibody was noticed. Following intramuscular injection of foreign protein with adjuvants, however, circulating antibody-antigen complex was demonstrated only subsequent to the appearance of free antibody. It persisted longer than in animals injected intravenously. Circulating antibody-antigen complex was present during the early phase of the immune response when a large dose of antigen was given intravenously. Only small amounts of free antibody were detected under these conditions. Apparently most of the antibody formed combined with the excess of free antigen present, leaving little or no free antibody in circulation.

Following intravenous administration of radioiodinated beef plasma albumin into non-immune rabbits, Knox and Endicott (18) compared the amount of antigen detected by precipitation by antibody with that measured by radioactivity determinations. They found good correlation between the two measurements until the 7th day after injection. By the 8th to the 10th day the sera had become negative for immunologically reactive antigen, while free antibody often was present. Nevertheless, a measurable amount of I^{131} persisted in the sera during this phase. Formation of antibody-antigen complex during antigen disappearance and antibody formation would explain this phenomenon. While free radioiodinated antigen that can be measured by precipitation with antibody had disappeared, some of the antigen might still have persisted in circulation as antibody-antigen complex. Such bound antigen would be detected by radioactivity measurements although it would not precipitate upon addition of further antibody *in vitro*. Dixon and coworkers (15, 2) failed to detect radioactivity in rabbit sera obtained beyond the 8th day of intravenous injection of radioiodinated beef plasma globulin. The dose of protein administered in their work was smaller than that given in the present experiments. Eisen, Sherman, and Pressman (19) reported a persistence of considerable amounts of intravenously injected radioiodinated antiovalbumin rabbit serum globulin in the blood of rats up to the 19th day. McMaster and Kruse (20), using a sensitive method for the detection of antigen, also found a prolonged persistence of antigen in mice.

The large amount of precipitable antibody-antigen complex found after injection of antigen with adjuvants is surprising since only 80 mg. of antigen was used for the primary injection, while 3 to 4 gm. was given in the case of rabbits injected intravenously. Perhaps the antibody-antigen complex precipitated from the serum following injection with adjuvants was formed in the presence of an excess of free antibody. As yet we have not been able to determine the ratio of antibody to antigen in the precipitates obtained. Differences in the ratio would explain the observations.

SUMMARY

Immune sera were subjected to treatment with alkali in the cold to cause dissociation of antigen-antibody complexes. Precipitates and a capacity to fix complement developed in some of these sera subsequent to such treatment.

The specific immunologic nature of these phenomena and their observation in relation to disappearance of free antigen and appearance of free antibody in circulation are discussed. The phenomena observed appear to be consistent with the assumption that a circulating antibody-antigen complex is revealed as a result of treatment.

BIBLIOGRAPHY

1. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. V., and Janeway, C. A., *J. Exp. Med.*, 1950, **91,** 505.
2. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *J. Immunol.*, 1951, **67,** 243.
3. Kendall, F. E., *J. Clin. Inv.*, 1937, **16,** 921.
4. Sternberger, L. A., and Maltaner, F., *Science*, 1951, **114,** 414.
5. Sternberger, L. A., and Pressman, D., *J. Immunol.*, 1950, **65,** 65.
6. Sternberger, L. A., and Petermann, M. L., *J. Immunol.*, 1951, **67,** 207.
7. Kleinschmidt, W. J., and Boyer, P. D., *J. Immunol.*, 1952, **69,** 257.
8. Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M., *J. Immunol.*, 1948, **60,** 383.
9. Wadsworth, A., Maltaner, E., and Maltaner, F., *J. Immunol.*, 1931, **21,** 313.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62,** 697.
11. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55,** 555.
12. Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97,** 405; **98,** 63.
13. Sternberger, L. A., *J. Am. Med. Assn.*, 1952, **150,** 1591.
14. Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1938, **35,** 93, 105, 217.
15. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, **10,** 553.
16. Wertheimer, E., and Stein, L., *J. Lab. and Clin. Med.*, 1944, **29,** 1082.
17. Lerner, A. B., *Am. J. Med. Sc.*, 1947, **214,** 410.
18. Knox, W. C., and Endicott, F. C., *J. Immunol.*, 1950, **65,** 523.
19. Eisen, H. N., Sherman, B., and Pressman, D., *J. Immunol.*, 1950, **65,** 543.
20. McMaster, P. D., and Kruse, H., *J. Exp. Med.*, 1951, **94,** 323.