STUDIES ON ANTIGEN–ANTIBODY COMPLEXES

I. ELIMINATION OF SOLUBLE COMPLEXES FROM RABBIT CIRCULATION*

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In vivo-formed antigen–antibody complexes cause transient glomerulonephritis and vasculitis in experimental animals when appropriate antigens are administered to unimmunized animals in a single dose (1, 2). Similar antigens administered in multiple doses induce chronic disease (1, 3). Antigen, antibodies, and complement components have been demonstrated in lesions produced in this manner. Furthermore, antigen–antibody complexes have been shown as causative agents of glomerulonephritis in human systemic lupus erythematosus (4), acute poststreptococcal glomerulonephritis (5), malaria (6), and other conditions. However, in experimental models and in human diseases, the characteristics of immune complexes that become entrapped by vascular and glomerular basement membranes to cause disease are not fully understood. In addition, detailed descriptions are not available on the characteristics of immune complexes that form in vivo from the native antibodies and the antigens that are introduced experimentally in animals or by disease processes in man. Similarly, quantitative information on the fate of such complexes is lacking.

When antigens are administered to immunized animals in small doses or if immune complexes are given to nonimmunized animals, the immune complexes are removed rapidly in part by the reticuloendothelial system (7, 8). Weigle (9) showed that if intravenously injected antigen–antibody complexes are prepared at equivalence or in slight antigen excess, they are quickly removed from the circulation of rabbits and that similar complexes prepared in large antigen excess persist longer. He concluded that the size of the complexes played a major role in their clearance from the circulation. Cochrane and Hawkins (10) also indicated that immune complexes greater than 19S are quickly removed from the circulation of guinea pigs and that small antigen–antibody complexes persist in the circulation. These investigators suggested that the immune complexes had to exceed a certain critical size and that vascular permeability had to be increased before complexes were entrapped by vascular or glomerular basement membranes. The half-life of the complexes that are rapidly removed has not been well defined and the role of the complement-fixing ability of these complexes has not been characterized fully.

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The current studies were undertaken to examine in rabbits the disappearance of intravenously administered soluble antigen-antibody complexes in relation to their size, complexity (number of antigen and antibody molecules in a complex), and complement-fixing ability. The presented data show that the rapidly cleared complexes fix complement well and consist of more than one antigen and two antibody molecules. Complexes composed of one antigen and two antibody molecules do not fix complement effectively and are removed slowly from the circulation. Furthermore, complexes prepared from antibodies altered by reduction and alkylation of interchain disulfide bonds do not fix complement effectively and their clearance from circulation is markedly retarded. However, the clearance of unaltered immune complexes was not affected by depletion of several complement components. These observations prompted the conclusion that complement fixation is not essential to the clearance of immune complexes, but the characteristics of complexes that permit complement fixation closely parallel those that predispose complexes to rapid clearance from the circulation due to uptake by the reticuloendothelial system.

Materials and Methods

Preparation of Antigens.—Several antigens were purified for immunization and preparation of antigen–antibody complexes. Unaggregated human serum albumin (subsequently designated HSA)[1] was obtained by gel filtration of crystallized human albumin (Mann Research Laboratories Inc., New York) with a column of Sephadex G-200 (5 $cm^2 \times 95$ cm volume; equilibrated with 0.2 M sodium borate, 0.15 M NaCl, pH 8.2). Only the symmetrical monomeric HSA peak was pooled for further use. The serum of a patient with Waldenström's macroglobulinemia was used as the source of the human γM-globulin for immunization and for preparation of immune complexes. The serum was first submitted to preparative electrophoresis (11) and then the fraction of the macroglobulin with the highest protein concentration was applied to a calibrated Sepharose 4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (5 $cm^2 \times 95$ cm volume, equilibrated with 0.2 M sodium borate, 0.15 M of NaCl, pH 8.2). The symmetrical peak of unaggregated γM-globulin with λ-chains was pooled for further use. Bence Jones proteins were isolated from the lyophilized urine of the same patient. This was achieved with preparative electrophoresis and gel filtration as described above. The symmetrical peak of the dimerized λ-chains was pooled for further use. Human γG-globulin was obtained from human fraction II (Mann Research Laboratories, New York) by gel filtration over Sephadex G-200 to remove aggregated material and contaminants. The purified antigens were used within 2 wk for preparation of antigen–antibody complexes; during this time they were stored at 4°C. The protein concentrations were determined by the Folin procedure.

Several New Zealand rabbits were immunized with each of the purified antigens for production of antibodies. The antigens were emulsified with complete Freund adjuvant and given subcutaneously or intramuscularly at weekly intervals in doses of 1–2 mg per rabbit for a minimum of 4 doses. Thereafter, weekly bleedings of 30–50 ml were obtained; the serum was harvested and stored at -20°C. In 4–6-wk intervals additional doses of the appropriate

[1] *Abbreviations used in this paper*: Anti-HSA (aHSA in figs.), antibodies to HSA; Anti-$HSA^{125}I$ red. and alk., reduced and alkylated anti-$HSA^{125}I$; CoF, cobra venom factor; HSA, human serum albumin.

antigens were administered and the bleedings were continued at 1–2-wk intervals. For each antigen the antisera were pooled from several rabbits.

Isolation of Antibodies.—The solid-phase immunoadsorbent technique of Axén and Porath was adapted for the preparation of sufficient amounts of purified antibodies (12). Sepharose 4B was washed with distilled water and with 0.2 M sodium borate, 0.15 M NaCl, pH 8.2; then the pH was brought to 11.0 with 1.0 M NaOH in a pH stat. Subsequently a solution of cyanogen bromide was added to provide 1 mg of CNBr for each milligram of dry Sepharose; the pH was maintained at 11.0 ± 0.2 for 20 min with 1.0 M NaOH. The activated Sepharose was washed in a fritted disc glass filter with copious amounts of cold distilled water and then with successive 100 ml portions of the cold borate buffer cited above. Finally a slurry of Sepharose was made and the protein to be coupled was added at a ratio of more than 2 mg of dry Sepharose to 1 mg of protein. This mixture was slowly tumbled by rotor at 4°C overnight. Subsequently, the Sepharose slurry was poured into a glass column, washed with the borate buffer, and then with a solution of 0.01 M HCl, 0.15 M NaCl. At each step the washing was carried out until no further protein was detected by absorbance at 280 mμ. After this, the column was washed with the borate buffer to remove all of the dissociating solution. The amount of protein recovered was determined and the amount of protein coupled to the Sepharose was calculated. In this manner columns of solid-phase immunoadsorbents were prepared with HSA, a Waldenström's macroglobulin, a λ-Bence Jones protein, and normal human γG-globulins.

The solid-phase immunoadsorbent columns were utilized for isolation of specific antibodies. In this procedure the appropriate antiserum was decomplemented by heating at 56°C for 30 min, cooled, and centrifuged at 1500 *g* to remove any insoluble material. After this, the antiserum was applied to the appropriate immunoadsorbent column at a flow rate of 10–20 ml/hr; the column was washed with the borate buffer cited above until the effluent contained no protein by 280 mμ absorbance. Thereafter, the antibodies were eluted with a solution of 0.01 M HCl, 0.15 M NaCl, or with 3.0 M sodium thiocyanate (13). The effluent fractions were collected and 280 mμ absorbance was determined. The protein-containing fractions were pooled immediately and dialyzed against cold borate buffer. Upon neutralization or removal of thiocyanate, some precipitate formed; this was separated by centrifugation. These preparations of eluted antibodies were either lyophilized from distilled water for storage or processed for further use.

Iodination of Antibodies.—The isolated antibodies were trace-labeled with ^{125}I with 1–2 moles of iodine per mole of protein, using the iodine monochloride method (14). After removal of unbound radioactivity by Dowex 1 × 4 in chloride phase or by dialysis, the protein solutions were applied to columns of G-200 Sephadex in borate buffer (same as above) to remove any high molecular weight antibodies or aggregated material. The symmetrical peak of unaggregated antibodies was pooled and used for preparation of complexes. Samples of the labeled antibodies were reduced and alkylated as previously described (15) before passage over G-200 Sephadex column. The same method of iodination was used to prepare labeled antigens.

Preparation and Characterization of Immune Complexes.—Once the ^{125}I-labeled antibodies, free of aggregates, had been obtained, quantitative precipitin curves were constructed to determine equivalence for each antibody preparation. To obtain soluble antigen–antibody complexes at a desired antigen excess, antibodies were added to antigen solutions. These preparations were allowed to incubate at 4°C for a minimum of 2 hr, but at times were stored at this temperature overnight. Before utilization for further studies they were centrifuged at 1500 *g* for 15 min.

The size and heterogeneity of antigen–antibody complexes was determined by linear sucrose density gradient ultracentrifugation (16). 0.40 ml specimens were applied to the gradients when using a Beckman-Spinco SW41Ti rotor (Beckman Instrument Co., Fullerton,

Calif.) and 0.20 ml specimens were applied when using a SW65Ti rotor. The gradients were harvested from the bottom and radioactivity in each fraction was assayed by an automatic well-type gamma counter. The radioactivity in each fraction was plotted as per cent of gradient volume (see Fig. 2). In this manner the separations from run-to-run could be easily compared and were shown to be highly reproducible. The unbound 6.6S antibodies served as a convenient marker to calculate the sedimentation coefficient of complexes. At times human γM- or rabbit γG-globulins were used as markers in the gradients.

The complement-fixing ability of immune complexes was determined by the method of Wasserman and Levine (17) as modified by Gilliland et al. (18) to yield a final volume of 3.5 ml in each tube. 1.5 CH_{50} units of guinea pig complement were used per tube and serial twofold dilutions of complexes were carried out. Complement-fixing ability was expressed in micrograms of antibody required to fix 50% of complement in this system. All preparations of complexes were dialyzed against a 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, 0.15 M NaCl buffer, pH 7.5, before testing for complement fixation to remove the sucrose or other buffers in which the complexes were prepared. The normal and depleted levels of rabbit complement were assayed by the methods cited above.

Administration of Complexes and their Disappearance from Rabbit Circulation.—The immune complexes, containing 2–4 mg of antibody, were administered intravenously to nonimmunized rabbits that had been pretreated with sodium iodide in drinking water for 1–2 days. The injections were given into the marginal vein of one ear and the samples were obtained from the other ear 5, 10, 30, and 60 min after the injection. Subsequent specimens were obtained at longer intervals. The experiments were usually terminated after 4 days to avoid the autologous newly formed antibodies from complicating the experiments. In preliminary experiments, rabbits were shown to start immune clearance of the antigen 5–6 days after the injection of immune complexes.

About 2.0 ml of blood was obtained at each bleeding. The clot was allowed to form; the serum was harvested and stored at 4°C for further studies. Accurately measured portions of serum were assayed for the presence of ^{125}I. Additional samples of serum from serial bleedings were diluted 1:4 with borate buffer and submitted to density gradient ultracentrifugation as described above.

Depletion of Complement Components.—To assess directly the role of complement components in the clearance of immune complexes from the circulation, rabbits were depleted of complement components by being given either heat-aggregated human γG-globulin or the purified anticomplementary cobra venom factor. The former was prepared according to the method of Christian (19) and the latter was purified by ion-exchange chromatography and gel filtration according to the procedures of Cochrane et al. (20). Further details of these preparations and their evaluation are given in another paper.[2] In order to suppress complement by the cobra venom factor (subsequently abbreviated CoF), the 3 kg rabbit was given 225 units of CoF intraperitoneally at 24, 20, and 18 hr before injection of complexes. 320 units of CoF were given intravenously 30 min before the complexes and thereafter once a day for the duration of the experiment. To suppress complement by aggregated human γG-globulin, 25 mg were given intraperitoneally 3 and 2 hr before the complexes, 5 mg intravenously at 1 hr, and 10 mg intravenously at 30 min before the complexes. Thereafter the rabbit received 25 mg intraperitoneally at 4 hr and subsequently twice a day throughout the experiment. Serum for complement assays was obtained before the next injection of agents for depletion of complement. These serum specimens were frozen within 1 hr at −70°C and stored until assayed for total hemolytic complement.

[2] Arend, W. P., and M. Mannik. Studies on antigen–antibody complexes. II. Quantification of tissue uptake of soluble complexes in normal and complement-depleted rabbits. Submitted for publication.

Analysis of Data.—The amount of radioactivity in sequential serum specimens was determined. The concentration of isotope per unit volume of serum at zero time (time of injection) was obtained by extrapolation of the 5, 10, and 15 min values, assuming that complete mixing has occurred at 5 min after injection. The zero time concentration was taken as 100% of the concentration of the injected dose. The per cent of remaining isotope was calculated for each bleeding and was plotted on semilogarithmic paper against time. In these calculations no allowance was made for the blood drawn from rabbits for analysis. Starting with the exponential component with the longest half-life, the graphic "peeling" method (21) was used to obtain an estimate of the half-life and the percentage of each exponential component as well as the number of such components. Some data were further analyzed by a CDC 6400 computer by the SAAM 22 program of Berman and Weiss (22). Each disappearance curve was tested for the estimated number of exponential components as well as for one more and one less exponential component. The goodness of fit was judged by the sum of squares of the differences between observed and calculated values, the estimates of parameter variance, and presence or absence of any progressive deviations of observed and calculated values.

RESULTS

Characteristics of Isolated Antibodies Used for the Preparation of Immune Complexes.—Several columns containing HSA coupled to 4B Sepharose were used repeatedly for isolation of antibodies to HSA (subsequently abbreviated anti-HSA). There was no apparent loss of the capacity of these columns to bind antibodies. During the elution of anti-HSA with 0.01 M HCl, 0.15 M NaCl, the antibodies emerged as the pH of the effluent dropped as indicated in Fig. 1. The eluted proteins were pooled and then neutralized by dialysis against borate buffer (see Materials and Methods). During this step 5–10% of the total protein precipitated. The nature of the precipitated material was not elucidated, but when ^{125}I-labeled HSA was coupled to Sepharose 4B, then radioactivity of the antigen was not recovered in the precipitate; this indicated that the precipitate did not contain antigen–antibody complexes and suggested that the precipitate represented denatured antibodies. After iodination with ^{125}I, the antibody preparations were passed over columns of Sephadex G-200 and the homogeneous protein peak that eluted as γG-globulin was utilized for the preparation of immune complexes. Immunoelectrophoresis of the antibody preparation after gel filtration disclosed only γG-globulin; furthermore, Ouchterlony plate analysis of the same material disclosed only one precipitin line with goat antisera against rabbit serum. Purified and labeled antibodies to the other antigens were similarly composed of only rabbit γG-globulin.

To determine that the biological half-life of the isolated antibodies was not altered by the elution procedures, two rabbits received simultaneously normal rabbit γG-globulin labeled with ^{131}I and anti-HSA labeled with ^{125}I. The serum disappearance curves of the two materials were superimposable and the graphically estimated half-lives were nearly identical for the γG-globulin and anti-HSA: 74 and 76 hr respectively in both rabbits. Similar half-lives were obtained for anti-γM labeled with ^{125}I.

Disappearance of HSA–Anti-HSA complexes.—Soluble complexes of HSA–

anti-HSA ^{125}I were prepared at fivefold antigen excess and portions of these complexes were analyzed by density gradient ultracentrifugation before injection into rabbits. Using the 6.6S peak of unbound antibodies as a reference point, the sedimentation coefficients of complexes were calculated. A discrete 11S peak of complexes was present (see Fig. 2); this was followed by a spectrum of complexes ranging from 14S to 22S (at 10% of gradient volume) and desig-

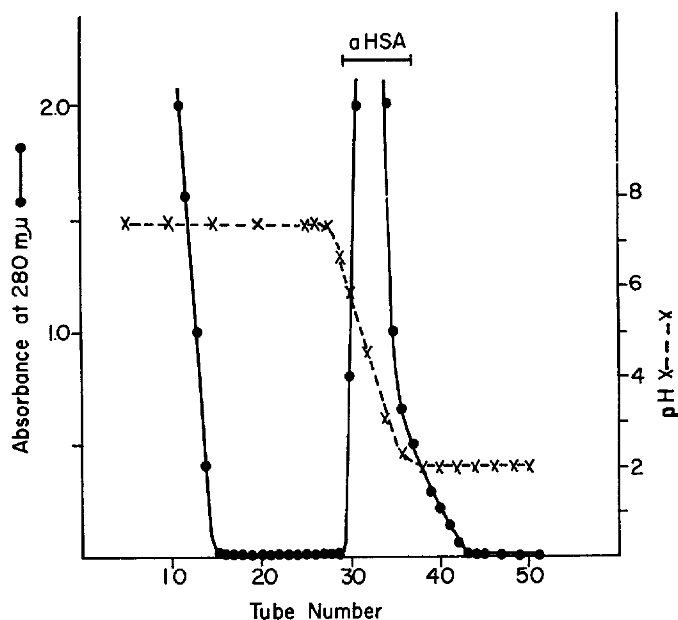

FIG. 1. Elution of anti-HSA (aHSA) from an HSA-Sepharose 4B column. 200 ml of decomplemented antiserum was applied to the column. After the serum had run through, the column was washed with borate buffer until no further protein eluted. At that time, elution with 0.01 M HCl, 0.15 M NaCl was started. As the pH of the effluent decreased, protein was eluted. The peak indicated with a bar was pooled for further study.

nated as >11S complexes. However, if a Waldenström's macroglobulin was used as a marker, the sedimentation coefficients for all the complexes were higher. In one experiment complexes were prepared from anti-HSA^{125}I and HSA^{131}I in fivefold antigen excess and analyzed on density gradients. The molar ratio of the antibody and antigen in the 11S peak was calculated from the specific activities of these materials, using the molecular weights of 145,000 (23) and 67,000 for the anti-HSA and HSA respectively. In the center of the 11S peak of complexes, the molar ratio of antibody to antigen approached 2 (2.05 and 1.89 in separate experiments). The molecular weight of the 11S peak

was estimated at 357,000 by the method of Martin and Ames (16). Therefore it was concluded that these complexes were composed of one HSA and two anti-HSA molecules.

Several density gradient separations were performed on HSA–anti-HSA^{125}I complexes at fivefold antigen excess and appropriate pools were made (illustrated in Fig. 2) to obtain sufficient >11S and 11S complexes for complement-fixation studies. The complexes at fivefold antigen excess contained 46% of

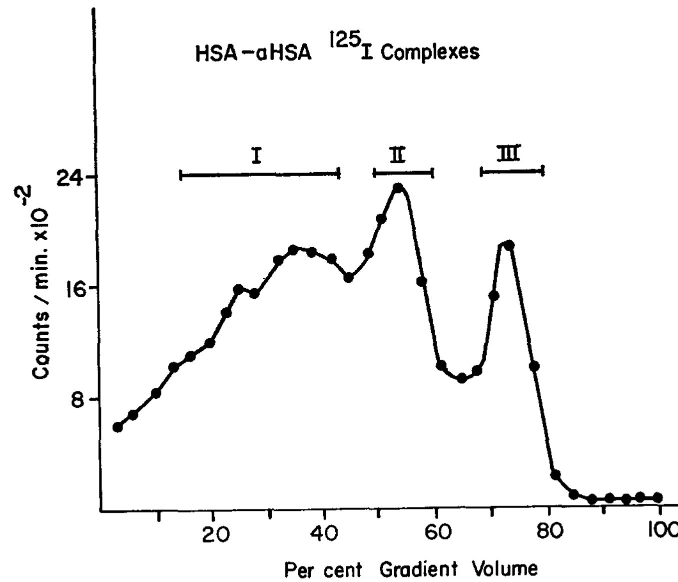

FIG. 2. Density gradient ultracentrifugation pattern of HSA–anti-HSA^{125}I complexes at fivefold antigen excess. A gradient of 10–30% sucrose and SW41Ti rotor were used; the top of the gradient is represented by 100% of gradient volume. Pool I represents >11S complexes that range from 14S to 22S, pool II, 11S complexes, and pool III, unbound 6.6S antibodies.

>11S complexes, 34% of 11S complexes, and 20% of 6.6S antibodies. The >11S complexes fixed complement effectively in that 0.095 μg of antibody was required to fix 50% of complement. The 11S complexes required 10 times more antibody protein (0.97 μg) to fix the same amount of guinea pig complement. The 11S complexes were unstable in the absence of excess antigen in that they reequilibrated to form some >11S complexes and some free 6.6S antibody as determined by repeated ultracentrifugation. Therefore it is likely that the complement-fixation data overestimated the ability of the 11S complexes to fix complement.

The soluble complexes of HSA–anti-HSA^{125}I, prepared at fivefold antigen excess on several occasions, were injected intravenously into seven rabbits.

The disappearance curves of these complexes were composed of three exponential components. The serum disappearance curves for two such rabbits are illustrated in Fig. 3. The two sets of experimental points and the corresponding curves calculated by computer were nearly superimposable. The half-lives of the exponential components and their percentages are recorded in Table I. In earlier

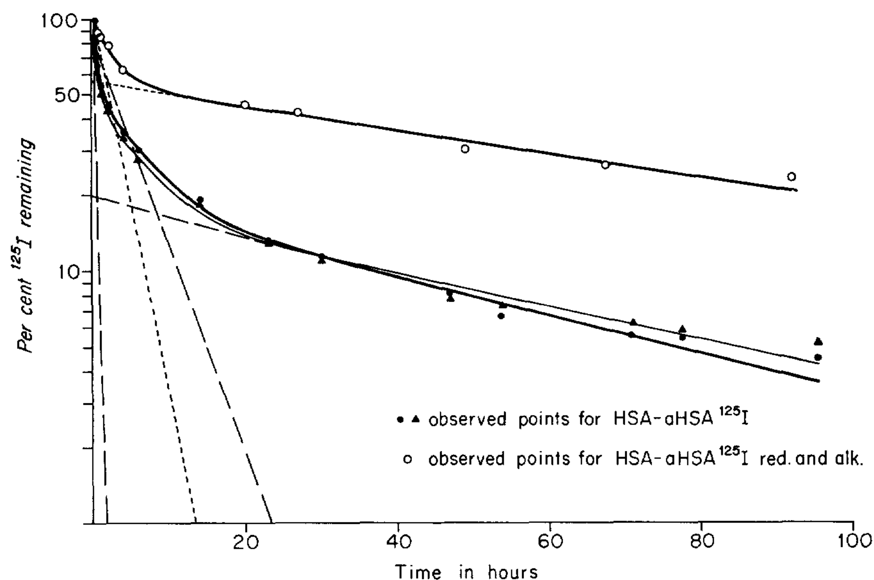

FIG. 3. Disappearance of HSA-anti-HSA^{125}I and HSA-anti-HSA^{125}I red. and alk. complexes from the circulation of rabbits. The solid circles (●) and the solid triangles (▲) indicate the experimentally observed points for the disappearance of HSA-anti-HSA^{125}I complexes in two rabbits. The solid lines indicate the curves fitted to these points by computer. The larger broken lines (– –) indicate the three exponential components that compose the curve indicated by the heavier solid line fitted to the experimental points marked by solid circles. The open circles (○) indicate the experimentally observed points for the disappearance of HSA-anti-HSA^{125}I red. and alk. complexes in one rabbit. The solid line indicates the curve fitted to these points by computer. The smaller broken lines (----) indicate the two exponential components that compose this curve.

experiments the values were graphically determined and similar values were obtained. The fastest exponential components had a mean half-life of 0.24 hr in seven experiments (range 0.16–0.37 hr) and 44.3% (range 40–51%) of the radioactivity was eliminated from the circulation with this half-life. The other two exponential components had longer half-lives. The second component had a mean half-life of 4.3 hr (range 2.8–7.5 hr), and 38.3% (range 32–40%) was eliminated from the circulation with this half-life. The third and slowest component had a mean half-life of 47 hr (range 40–65 hr) and 17% (range 13–20%)

of radioactivity was eliminated from circulation with this half-life. The half-life of HSA^{125}I alone was 57 hr and the equilibration phase (first exponential component) had a half-life of 2.3 hr.

Sequential serum specimens, obtained from rabbits in whom the disappearance of complexes was studied, were submitted to density gradient ultracentrifugation. The >11S complexes were quickly removed (see Fig. 4). Smaller complexes and the 6.6S antibodies clearly persisted longer in the circulation. To determine the half-life of the complexes of different sizes, counts remaining

TABLE I

Disappearance of HSA-Anti-HSA Complexes from the Circulation: Half-Lives of Exponential Components and the Percentages of Antibodies in each Component

Nature of complexes administered	Rabbit No.	First (fastest) component		Second component		Third component	
		t ½*	Per cent*	t ½*	Per cent*	t ½*	Per cent*
		(hr)		*(hr)*		*(hr)*	
HSA-anti-HSA^{125}I; 5-fold Ag excess	Mean values and ranges for five rabbits	0.25 (0.16–0.37)	45 (40–51)	4.6 (2.8–7.5)	38 (32–40)	49 (40–65)	17 (13–20)
HSA-anti-HSA^{125}I; 5-fold Ag excess	M30	0.21 ± 0.02	41.0 ± 2.0	3.59 ± 0.47	39.8 ± 2.3	40.0 ± 5.6	19.0 ± 2.2
HSA-anti-HSA^{125}I; 5-fold Ag excess	M31	0.24 ± 0.02	44.5 ± 2.5	3.83 ± 0.61	37.9 ± 2.7	47.8 ± 9.6	17.0 ± 2.4
HSA-anti-HSA^{125}I red. & alk.; 5-fold Ag excess	Mean values and ranges for two rabbits	2.1 (2.0 & 2.2)	39 (38 & 40)	63 (57 & 69)	61 (60 & 62)		
HSA-anti-HSA^{125}I red. & alk.; 5-fold Ag excess	M14	1.98 ± 0.28	43.2 ± 2.8	63.0 ± 6.9	54.2 ± 2.8		

* In experiments analyzed by computer, 1 SD of variance is given. For other experiments the graphically estimated values are given with the mean and the range of values.

under each of the components were estimated and plotted directly on semilogarithmic paper against time (see Fig. 5). In this manner the half-lives of the >11S, 11S, and 6.6S materials were estimated. The half-life of the >11S components was 0.16–0.28 hr and these curves were straight lines on semilogarithmic plots and were therefore single exponential components. The disappearance curve of the 11S complexes was composed of two exponential components, and these were thought to represent equilibration and catabolism. The catabolic phase of 11S complexes and the 6.6S antibodies appeared to parallel each other.

HSA–anti-HSA^{125}I complexes were prepared at 20-fold antigen excess. This preparation was analyzed by density gradient ultracentrifugation as described above. In these complexes 32% of the radioactivity sedimented as >11S complexes and 48% as 11S complexes. When these complexes were injected

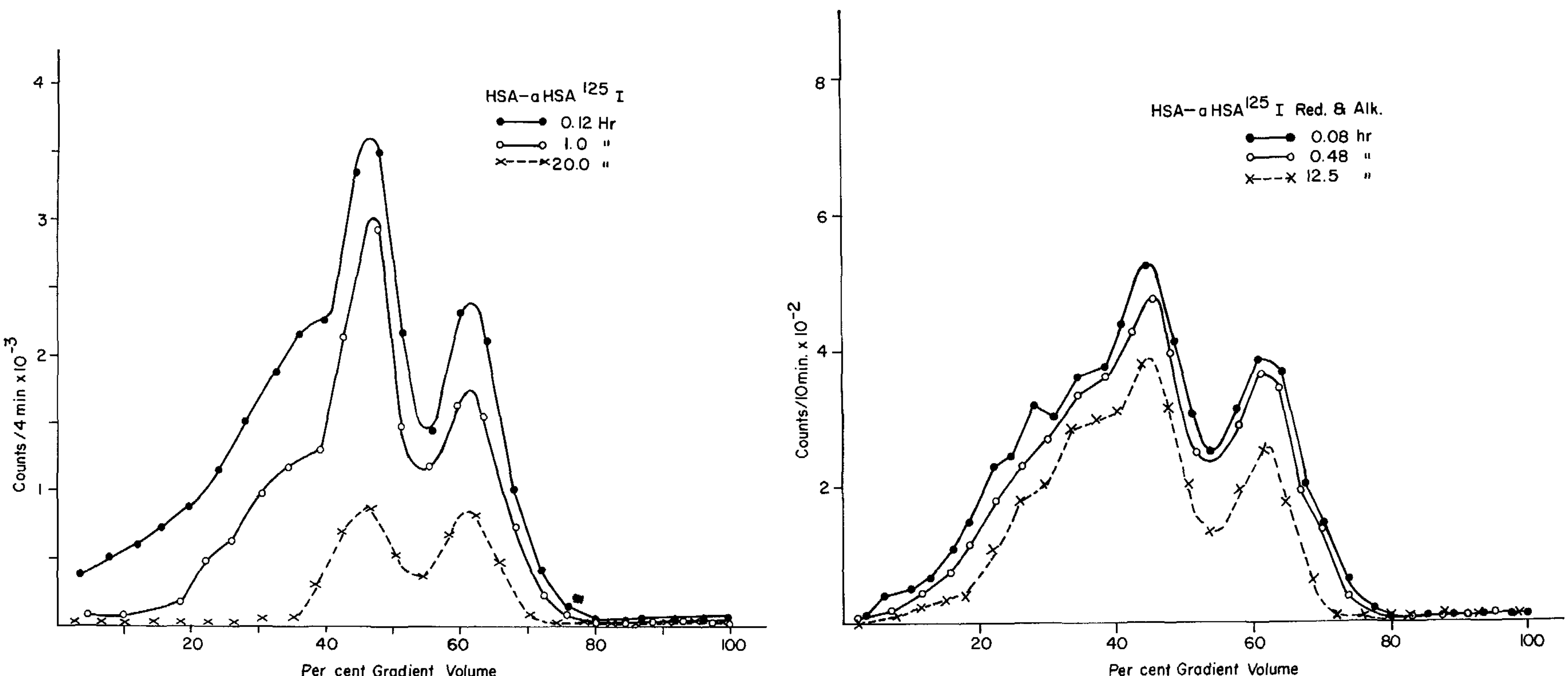

FIG. 4. Density gradient ultracentrifugation patterns of sequential sera of rabbits who had received immune complexes. Gradients of 10–40% sucrose and SW65Ti rotor were used; the top of the gradient is represented by 100% of gradient volume. On the left serum specimens of a rabbit which received HSA-anti-HSA^{125}I complexes show early disappearance of >11S complexes. In contrast, on the right the serum specimens of a rabbit which received HSA-anti-HSA ^{125}I red. and alk. complexes show the parallel decrease of >11S and 11S complexes and 6.6S antibodies.

into a rabbit, the disappearance curve showed three exponential components similar to previous experiments, but the per cent of the fastest component was decreased. On graphic analysis the fastest component had a half-life of 0.40 hr;

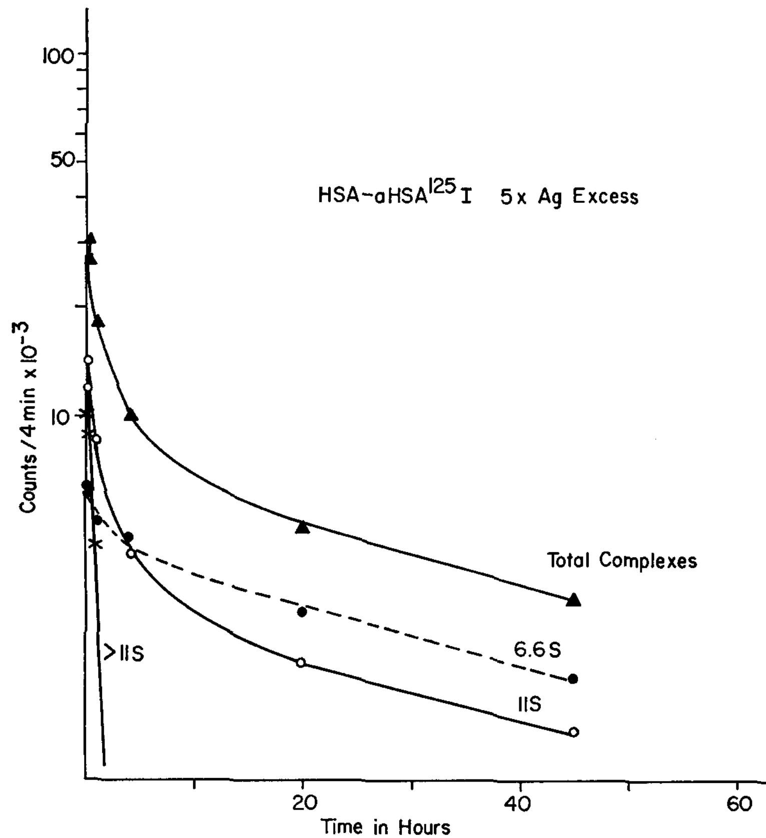

FIG. 5. Disappearance curves of the individual components of HSA-anti-HSA ^{125}I complexes prepared at fivefold antigen excess. The counts per 4 min remaining under the >11S and 11S complexes and 6.6S peak on density gradient ultracentrifugation were estimated for six bleedings and plotted against time. The >11S complexes (X——X) disappeared quickly as one exponential component, whereas the 11S complexes (O——O) and the 6.6S antibodies (●---●) had two exponential components.

34% of radioactivity was eliminated with this half-life. The second and third exponential components had half-lives comparable to the complexes prepared at fivefold antigen excess. Thus in this experiment the >11S material was decreased and a corresponding decrease was observed in the first component of the disappearance curve of the immune complexes.

Reduction and alkylation of interchain disulfide bonds of antibodies has been shown to decrease markedly the efficiency of these molecules to fix complement when they interact with the specific antigen (24). For this reason complexes were prepared at fivefold antigen excess from HSA and reduced and alkylated anti-HSA^{125}I (subsequently designated anti-HSA^{125}I red. and alk.). Density gradient ultracentrifugation of these complexes showed that $>$11S and 11S complexes and 6.6S antibodies were present in proportions similar to those in the experiments with anti-HSA^{125}I. The complexes prepared from reduced and alkylated antibodies were virtually unable to fix complement in that more than 63 μg was required to fix 50% of complement in the system employed. When these complexes were administered intravenously, their disappearance from the circulation was composed of only two exponential components (see Fig. 3 and Table I). The first component had a mean half-life of 2.1 hr (range 2.0–2.2 hr) and 40.3% (range 38–43%) of the radioactivity was eliminated from circulation at this half-life. The second component had a mean half-life of 63 hr (range 57–69 hr) and 58.7% of radioactivity was eliminated with this half-life. Density gradient centrifugation on sequential serum samples showed that the $>$11S, 11S, and 6.6S components disappeared from the circulation in parallel without a rapid loss of the $>$11S complexes as illustrated in Fig. 4 (right).

Disappearance of λ–Anti-λ and γG–anti-γG Complexes.—To explore the disappearance of immune complexes with other antigens, dimeric human λ-Bence Jones proteins (molecular weight of about 40,000) and human γG-globulin were chosen as antigens. The antibodies were isolated, labeled with ^{125}I, and processed in the same manner as described for the anti-HSA. The labeled antibodies to a λ-Bence Jones protein will be designated anti-λ^{125}I and the antibodies to γG-globulin will be designated as anti-γG ^{125}I. The latter predominantly contained antibodies to the Fc fragments. In addition, samples of antibodies to both antigens were reduced and alkylated.

Only 40% of the 6.6S anti-λ^{125}I and reduced and alkylated anti-λ^{125}I preparation precipitated at equivalence with the λ-Bence Jones proteins used as the antigen; the remaining 60% was not bound to antigen, as shown by density gradient centrifugation. Presence of idiotypic antibodies (25) could not account for the failure of precipitation since the same Bence Jones protein was used for immunization, isolation of antibodies, and preparation of complexes. Soluble complexes at 10-fold antigen excess were prepared with anti-λ^{125}I and with reduced and alkylated anti-λ^{125}I. Density gradient ultracentrifugation showed that these complexes were composed of $>$9S complexes, a discrete peak of 9S complexes, and 6.6S antibodies. In these complexes 20% of antibodies were in the $>$9S complexes. The λ–anti-λ^{125}I complexes fixed complement well and the λ–anti-λ^{125}I reduced and alkylated complexes fixed no complement. The above-mentioned complexes were given intravenously to separate rabbits.

The disappearance curve of the complexes with intact antibodies was composed of three exponential components (see Table II); the fastest component had a half-life of 0.09 hr and 19% of the labeled antibodies were removed from the circulation with this half-life. The disappearance curve of the complexes with reduced and alkylated antibodies was composed of only two exponential components (see Table II). The initial rapid removal of complexes was clearly absent when the complexes were prepared with reduced and alkylated antibodies.

TABLE II

Disappearance of Other Immune Complexes from the Circulation: Half Lives of Exponential Components and the Percentages of Antibodies in Each Component

Nature of complexes administered	Rabbit No.	First (fastest) component		Second component		Third component	
		t ½*	Per cent*	t ½*	Per cent*	t ½*	Per cent*
		(hr)		*(hr)*		*(hr)*	
λ-anti-λ^{125}I; 10-fold Ag excess	M11	0.09 ± 0.00	18.9 ± 2.5	1.45 ± 0.20	40.8 ± 2.2	27.7 ± 2.1	40.7 ± 1.9
λ-anti-λ^{125}I red. & alk; 10-fold Ag excess	M29	1.20 ± 0.57	18.0 ± 3.3	38.5 ± 2.5	81.9 ± 3.0		
γG-anti-γG^{125}I; 5-fold Ag excess	M33	0.26 ± 0.02	37.0 ± 1.5	5.96 ± 0.66	39.4 ± 1.7	81.5 ± 9.8	24.0 ± 1.8
γG-anti-γG^{125}I; 5-fold Ag excess	M36	0.16 ± 0.03	66.5 ± 1.3	4.08 ± 0.66	25.7 ± 1.6	43.3 ± 12.2	7.8 ± 1.7
γG-anti-γG^{125}I red. & alk.; 5-fold Ag excess	M34	1.00 ± 0.16	40.1 ± 2.7	43.4 ± 3.0	61.3 ± 2.5		
γG-anti-γG^{125}I red. & alk.; 5-fold Ag excess	M37	1.9 ± 0.19	51.0 ± 2.2	41.5 ± 2.8	48.8 ± 2.1		
γM-anti-γM^{125}I; 5-fold Ag excess	M20	0.20 ± 0.01	59.8 ± 1.3	6.48 ± 1.23	33.5 ± 3.4	49.6 ± 33.9	7.7 ± 3.9
γM-anti-γM^{125}I red. & alk.; 5-fold Ag excess	M21	0.25 ± 0.04	31.1 ± 2.5	4.20 ± 0.59	47.6 ± 3.4	49.5 ± 11.4	22.4 ± 3.1

* These experiments were analyzed by computer, 1 SD of variance is given.

Two separate purifications of antibodies to human γG-globulin were carried out. Anti-γG^{125}I and reduced and alkylated anti-γG^{125}I were prepared from each purified batch. With the first batch, 60–65% of antibodies precipitated at equivalence and with the second batch, 80% of antibodies precipitated at equivalence. Soluble complexes at fivefold antigen excess were prepared with all four preparations of antibodies. On density gradient ultracentrifugation the complexes with intact and reduced and alkylated antibodies gave patterns of >13S, 13S, and 10S complexes and 6.6S antibodies. The 6.6S peak was used as a marker for the calculation of sedimentation coefficients. The amount of >13S complexes varied with the two preparations of antibodies. In the first prepara-

tion of antibodies (used for rabbits M33 and M34), 39% of antibodies were in the >13S fractions and in the second preparation of antibodies 67% were in the >13S fractions. The 13S complexes were estimated to have a molecular weight of 401,000 and the molar ratio of antibody to antigen approached 0.5. Therefore, these complexes were thought to be composed primarily of one antibody and two antigen molecules. The 10S complexes had a molecular weight of 270,000 and therefore were composed of one antibody and one antigen molecule. A

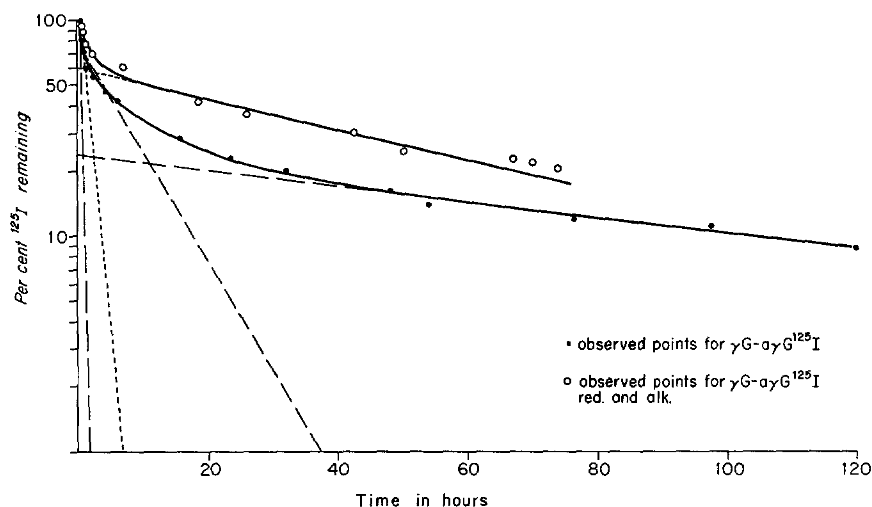

FIG. 6. Disappearance of γG–anti-γG^{125}I (aγG) and γG–anti-γG^{125}I red. and alk. complexes from the circulation of rabbits M33 and M34. The solid circles (●) and open circles (○) indicate the experimentally observed points for the disappearance of γG–anti-γG^{125}I and γG–anti-γG^{125}I red. and alk. complexes respectively; the solid lines, indicate the curves fitted to these points by computer. The larger broken lines (– –) indicate the three exponential components that compose the curve for the γG–anti-γG^{125}I complexes, and the smaller broken lines (---) indicate the two exponential components that compose the curve for the γG–anti-γG^{125}I red. and alk. complexes.

preparation of the >13S fraction of complexes, obtained from the γG–anti-γG^{125}I complexes made with the first batch of antibodies, required 0.18 μg of antibodies to fix 50% of guinea pig complement. The 13S and 10S complexes required 15 μg of antibodies to fix the same amount of complement. The complexes prepared from reduced and alkylated antibodies at fivefold antigen excess were given intravenously to rabbits; the complexes were from the first antibody preparation to M33 and M34 and from the second preparation to M36 and M37. With the intact antibodies the disappearance curve of γG–anti-γG^{125}I complexes was composed of three exponential components (see Table II and Fig. 6). The first and fastest component had a half-life of 0.26 hr

in M33 and 0.16 hr in M36; 40.1 and 66.5% of the radioactivity was eliminated from the circulation of these rabbits, respectively, at the fast half-lives. These percentages are close to the amounts of >13S complexes in each of the preparations. In addition, density gradient separation on sequential serums of M36 showed the rapid elimination of >13S complexes and persistence of 13S and 10S complexes and 6.6S antibodies. In contrast, when the complexes with reduced and alkylated antibodies were administered to rabbits M34 and M37 (see Table II), the disappearance curves were composed of two exponential components and the fastest component was absent. Furthermore, density gradient separations on sequential serum specimens showed the persistence of >13S complexes along with the smaller components.

Disappearance of γM–Anti-γM Complexes.—In order to evaluate the disappearance of even larger soluble immune complexes, the isolated Waldenström's macroglobulin of a patient was used as the antigen. All preparations of this macroglobulin contained some aggregates with sedimentation coefficients of 25S and 29S. The antibodies were prepared in the same manner as already described; they will be designated anti-$\gamma M^{125}I$ and the antibodies with reduced and alkylated interchain disulfide bonds will be designated anti–$\gamma M^{125}I$ reduced and alkylated. At equivalence 92% of these antibodies were precipitated. Antigen–antibody complexes were prepared at fivefold antigen excess and characterized by density gradient ultracentrifugation. These complexes were composed, using the 19S Waldenström's macroglobulin as a marker for calculation of sedimentation coefficients, of >25S, 25S, and 20S complexes, representing 58%, 21%, and 15%, respectively, of the labeled antibodies. The 25S complexes were not always clearly identifiable in the preparations before injection but became clearly evident and persisted when administered to rabbits (cf. Figs. 7 and 9). Furthermore, before injection a 20S peak of antibodies was present, but this component was never seen in rabbit circulation. A small peak of 6.6S antibodies was present. If the latter was used as a reference point to calculate the sedimentation coefficients, the 25S peak calculated to be 17S according to the methods of Martin and Ames (16). The concentration of sucrose was checked by refractometry in the fractions of the gradient and the gradients were found to be linear, except in the top 5–8% of gradient volume. It was not clear why different values were obtained with the two reference points. The 25S complexes were estimated to have a molecular weight of 1,360,000 and thus were thought to be composed of one antigen and three antibody molecules. Pools of the various sizes of complexes were made and tested for their ability to fix complement. The >25S complexes, ranging in size from 31–40S, required 0.175 μg of antibody to fix 50% of complement. The 25S complexes, which also contained some larger complexes (see Fig. 7), required 1.2 μg of antibody to fix the same amount of complement; the 20S complexes required 3.3 μg of antibody to fix 50% of complement. The complexes prepared from reduced and alkylated

antibodies were ineffective in complement fixation in that more than 43 μg of antibodies was needed to fix 50% of complement.

The soluble complexes prepared at fivefold antigen excess with the intact and reduced antibodies were given to separate rabbits intravenously. With the intact antibodies the disappearance curve of the complexes was composed of three exponential components (see Fig. 8 and Table II). The first and fastest component had a half-life of 0.20 hr and 60% of the radioactivity was elimi-

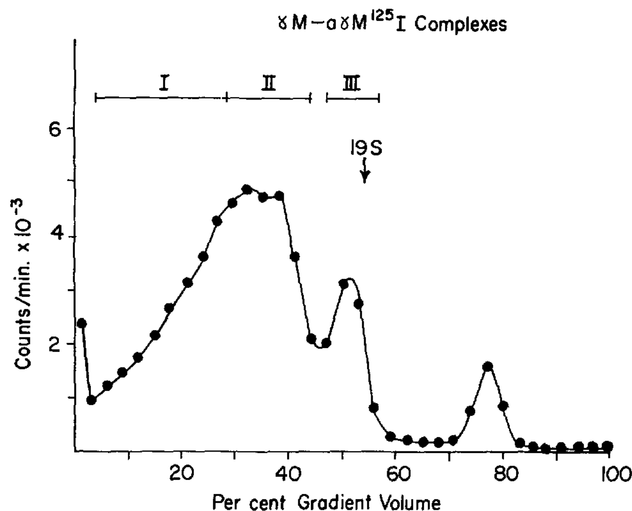

FIG. 7. Density gradient ultracentrifugation pattern of γM-anti-γM^{125}I (aγM) complexes at fivefold antigen excess. A gradient of 10–50% sucrose and SW41Ti rotor were used; the top of the gradient is represented by 100% of gradient volume. Pool I represents complexes from 31 to 40S, pool II, primarily 25S, and pool III, 20S complexes. The first point on the left represents the counts in the pellet. The arrow indicates the position of 19S γM-globulin marker.

nated with this half-life. This percentage was close to the amount of >25S antibodies in the γM-anti-γM^{125}I preparations. Furthermore, density gradient separation on sequential specimens of rabbit M20 showed the rapid elimination of >25S complexes and persistence of the 25S complexes and 6.6S antibodies (see Fig. 9). Of note is that in the earliest serum specimens the proportion of pelleted material had increased in comparison to the preinjection separation of the complexes. Furthermore, free iodine appeared in the 1–4-hr specimens as evidence of degradation of the rapidly eliminated material. For plotting of the serum disappearance curves, only the protein-bound radioactivity was used.

The complexes prepared with reduced and alkylated anti-γM^{125}I also had a disappearance curve composed of three exponential components. The first

component had a half-life of 0.25 hr, and 31% of the radioactivity was eliminated from circulation with this half-life. The presence of the rapid component in the disappearance of these complexes prepared with reduced and alkylated antibodies is unique in that it was not seen with the reduced and alkylated antibodies to HSA, λ-chains, and γG-globulins. Density gradient separation of

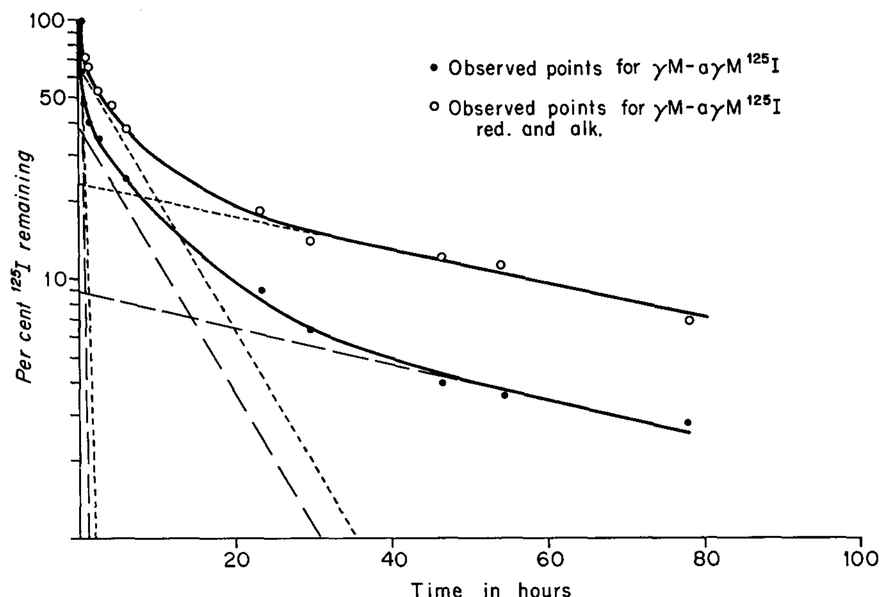

FIG. 8. Disappearance of γM-anti-γM^{125}I (aγM) and γM-anti-γM^{125}I red. and alk complexes from circulation of rabbits. The solid circles (●) and the open circles (○) indicate the experimentally observed points for the disappearance of γM-anti-γM^{125}I and γM-anti-γM^{125}I red. and alk. complexes respectively; the solid lines indicate the curves fitted to these points by computer. The larger broken lines (– –) indicate the three exponential components that compose the curve for the γM-anti-γM^{125}I complexes, and the smaller broken lines (---) indicate the three exponential components that compose the curve for the γM-anti-γM^{125}I red. and alk. complexes.

sequential serum specimens from this rabbit also disclosed the rapid elimination of >25S material; the 25S material persisted along with a shoulder of >25S complexes. The initial rapid elimination of γM–anti-γM^{125}I reduced and alkylated complexes was not due to rapid elimination of antigen alone, because when the antigen was labeled with ^{125}I and administered to a rabbit, the 19S material had a catabolic half-life of 76 hr. Only the aggregated γM-globulin was quickly removed. Partial aggregation of the antigen molecules before preparation of complexes could have contributed to the rapid clearance of some complexes with reduced and alkylated antibodies.

Experiments were conducted to determine the elimination of the immune complexes prepared from the same anti-$\gamma M^{125}I$ preparations but using as antigen the subunits of the γM-globulin prepared by reduction and alkylation (26). However, the γM-globulin subunits were quickly eliminated from circulation and because of this all complexes prepared with this antigen were also quickly removed.

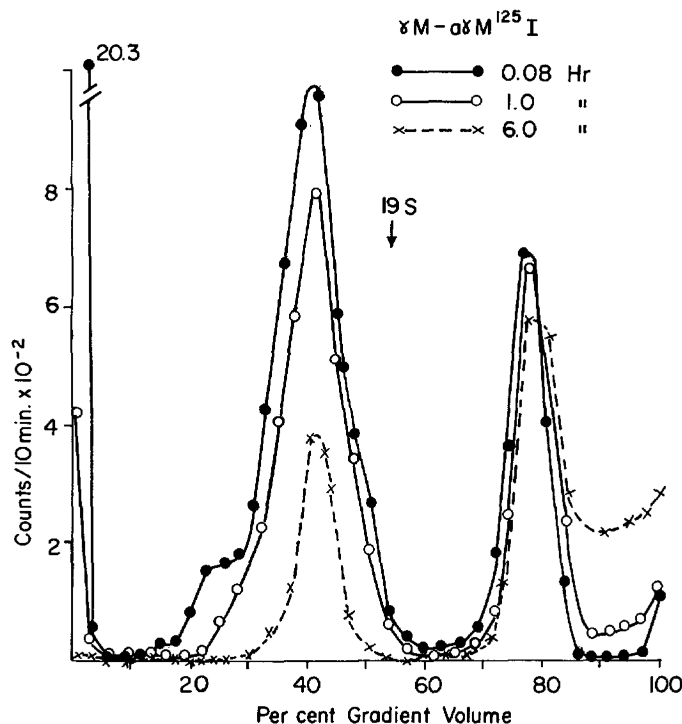

FIG. 9. Density gradient ultracentrifugation patterns of sequential sera of the rabbit that had received γM-anti-$\gamma M^{125}I$ (aγM) complexes. Gradients of 10–50% sucrose and SW41Ti rotor were used. The >25S complexes are quickly eliminated and the 25S complexes persist. The first points on the left represent the radioactivity recovered in the pellets.

Disappearance of HSA–Anti-HSA Complexes from Complement Depleted Rabbits.—The experiments described thus far were consistent with two possibilities: (*a*) complement fixation was necessary for rapid removal of circulating immune complexes of sufficient complexity; (*b*) rapid removal of immune complexes of sufficient complexity did not require complement, but this property was altered by reduction and alkylation of the interchain disulfide bonds of antibodies. To distinguish between these two possibilities, rabbits were depleted of complement components, the HSA–anti-$HSA^{125}I$ complexes at

fivefold antigen excess were administered, and serum specimens were obtained and analyzed for disappearance of complexes, as already described.

Treatment with CoF was started 24 hr before the complexes were given to depress the complement level. The complement level remained below 2 CH_{50} units throughout the experiment, except at the last bleeding (at 96 hr) the complement level was 2 units. In this rabbit the disappearance of the HSA–anti-$HSA^{125}I$ complexes was indistinguishable from that observed in normal rabbits. Three exponential components were present and the fastest component had a half-life of 0.18 ± 0.01 hr; $46.7 \pm 1.6\%$ of antibodies were removed with this half-life (for comparison see Table I).

The aggregated human γG-globulin was administered before the complexes to reduce the complement level and the injections were continued, as outlined in Materials and Methods, to keep the complement depressed. The normal complement was 27 CH_{50} units before the aggregates were given but was undetectable before the complexes were administered. However, 8 hr after the complexes were administered, the complement level had increased to 14 CH_{50} units and then gradually returned to 27–30 CH_{50} units in spite of the continued administration of the aggregates. In this rabbit the disappearance curve of the HSA–anti-$HSA^{125}I$ complexes was identical to that observed in normal rabbits. The curve was composed of three exponential components and the fastest component had a half-life of 0.21 ± 0.01 hr; $52.0 \pm 1.4\%$ of antibodies were removed with this half-life. The complement level did not remain depressed sufficiently throughout this experiment but it was undetectable through at least four half-lives of the first component. Therefore, it seemed safe to conclude that complement depletion with the CoF or the aggregated human γG-globulin did not alter the clearance of immune complexes from the circulation; in particular the rapid initial phase of the clearance of the $>11S$ HSA–anti-HSA complexes was not altered. This conclusion was strongly supported by data in another paper[2] showing that the uptake of immune complexes by the reticuloendothelial system was not diminished by complement depletion.

DISCUSSION

Soluble antigen–antibody complexes were prepared in fivefold antigen excess with specific isolated rabbit antibodies to antigens of varying size. The isolated antibodies were all γG-globulins and therefore were of constant size. The immune complex preparations with each of the antigens contained complexes of variable sizes because of the increasing number of antigen and antibody molecules involved. However, by virtue of antigens being of variable sizes, immune complexes of different sizes were obtained that contained comparable numbers of antigens and antibodies. The size of the soluble complexes was characterized on linear sucrose density gradients by taking advantage of the ^{125}I label on the antibodies. The soluble complexes in all systems (anti-HSA, anti-λ–chains, anti-

γG, and anti-γM) had characteristic peaks of antigen–antibody complexes that appeared as the smallest stable complexes of the antibodies and the respective antigens, ranging from 9S for the λ–anti-λ complexes to 25S for the γM–anti-γM complexes, with 11S for the HSA–anti-HSA and 13S for the γG–anti-γG complexes. These complexes which were isolated in three systems (anti-HSA, anti-γG, and anti-γM) were found to be ineffective in complement fixation. The residual complement fixation was thought to be due to rearrangement of antigens and antibodies after the removal of excess antigen, and this was actually demonstrated with HSA–anti-HSA for the 11S complexes. The 11S complexes in the HSA–anti-HSA system consisted of one antigen and two antibody molecules ($AgAb_2$). The Ag_2Ab complexes in this system would have a sedimentation coefficient of 10.2S. Small amounts of such complexes could have existed, but sucrose density gradient experiments disclosed no peak corresponding to such complexes. HSA possesses several different antigenic determinants that have been recognized by precipitating antibodies (27). The $AgAb_2$ complexes in this system must be thermodynamically more stable than the theoretically simpler and smaller Ag_2Ab complexes. Since the HSA is a multivalent antigen with several different antigenic determinants, it is possible that each of the antibodies in these complexes was directed to different antigenic determinants. The 13S complexes in the γG–anti-γG system were calculated to consist of Ag_2Ab. The 9S material in the λ–anti-λ system was estimated to consist of Ag_2Ab complexes also. The 25S complexes in the γM–anti-γM system were estimated to contain $AgAb_2$ to $AgAb_3$ complexes.

The observation that the $AgAb_2$ complexes did not fix complement is in agreement with the results of Hyslop et al. (28) who fractionated soluble complexes composed of antibodies and antigens with limited valence. They concluded from complement-fixation experiments and electron microscopic studies that soluble complexes with two antibodies did not fix complement and that complexes with four or more antibodies were able to fix complement. Our observations and the results of Hyslop et al. (28) are not in conflict wih the conclusion of Cohen (29). He studied complement fixation at equivalence with unaltered antibodies and with chemically altered antibodies that were unable to fix complement; he reached the conclusion that in the lattice of immune complexes two γG-globulin molecules had to be adjacent to each other to initiate complement fixation. In the $AgAb_2$ complexes the Fc regions of the antibodies appeared sterically apart under electron microscopy (28), and this may well account for their failure to fix complement.

In the antigen–antibody systems studied, at fivefold antigen excess, larger complexes were also present. These ranged from 14–22S in the HSA–anti-HSA complexes, designated as $>11S$ complexes, and these complexes fixed complement well. By virtue of their larger size, the $>11S$ complexes were thought to contain more antigen and antibody molecules than the $AgAb_2$ complexes. Cor-

respondingly larger complexes were present in the other systems as well and they fixed complement efficiently.

The antigen–antibody complexes that contained more than two antibody molecules (i.e. more complex than $AgAb_2$) were quickly removed from the circulation, the half-lives ranging from 0.16 to 0.40 hr (see Tables I and II). The proportion of antibodies removed quickly from the circulation corresponded closely to the percentage of antibodies found in the >9S, >11S, >13S, and >25S complexes respectively in the anti-λ, anti-HSA, anti-γG, and anti-γM systems. The rapid removal of these complexes was confirmed by density gradient ultracentrifugation of sequential serum samples. Furthermore, in the HSA–anti-HSA system complexes were prepared at higher antigen excess with a smaller proportion of antibodies in the >11S complexes and a correspondingly smaller proportion of antibodies was removed quickly from circulation. Weigle had similarly observed that with increasing antigen excess a larger proportion of injected antibodies persisted in circulation (9). In contrast, the antigen–antibody complexes composed of $AgAb_2$ (e.g. HSA–anti-HSA and γM–anti-γM) or Ag_2Ab (e.g. γG–anti-γG) persisted much longer in the circulation. These were the 11S complexes in the HSA–anti-HSA, 13S complexes in the γG–anti-γG and 25S complexes in the γM–anti-γM systems. Thus, the physical size alone in the limits of these experiments did not seem to dictate rapid removal of immune complexes. The rapidly removed complexes in the HSA–anti-HSA system ranged from 14S to 22S, yet in the γM–anti-γM system 25S complexes persisted. The rapidly removed complexes fixed complement well in all systems examined in these experiments.

In the λ–anti-λ, HSA–anti-HSA, and γG–anti-γG systems, the first and rapid removal of complexes was eliminated when the interchain disulfide bonds of antibodies were reduced and alkylated. This alteration of antibodies rendered them ineffective in complement fixation when bound with antigens. As already mentioned, the $AgAb_2$ and Ag_2Ab complexes that persisted in the circulation were ineffective in complement fixation. Together these observations suggested initially that complement fixation was important in rapid clearance of immune complexes from the circulation (30). However, if this were true, then depletion of complement should alter the clearance of immune complexes from the circulation. Depletion of complement components with cobra venom factor or with aggregated human γG-globulin during the phase of rapid clearance of complexes did not alter the elimination of HSA–anti-HSA complexes. This observation was further substantiated by measuring the quantitative uptake of immune complexes by the reticuloendothelial system in normal and complement-depleted rabbits during the rapid phase of clearance of immune complexes.[2] Therefore, the conclusion was reached that complement fixation is not essential for the clearance from circulation of immune complexes that are composed of γG-globulins as antibodies. Yet the clearance of these complexes was markedly

altered by cleavage of interchain disulfide bonds, which also diminishes complement fixation. This alteration of antibody molecules therefore appeared to change two separate functions of the γG-globulin molecules. The most important parameter for rapid clearance of immune complexes seemed to be their complexity, in that they had to contain more than two antibody molecules.

In experiments where bovine serum albumin or HSA was administered to rabbits for production of acute or chronic glomerulonephritis (10, 3), circulating immune complexes were observed that corresponded in size to the 11S complexes observed in our HSA–anti-HSA system. However, in some rabbits $>19S$ complexes were seen in these experiments also, but the characteristics of antibodies in these complexes were not defined. The results presented in this study show that the $>11S$ complexes of HSA–anti-HSA (γG-globulin antibodies) are quickly cleared from the circulation and therefore only serum specimens obtained shortly after administration of antigen to immunized animals can be expected to contain detectable immune complexes of this degree of complexity. Soluble $AgAb_2$ complexes can also be achieved with antigens of limited valence. The persistence of such complexes in circulation has been demonstrated (31). Clearance of soluble immune complexes from the circulation of decomplemented animals has not been examined previously. However, Spiegelberg et al. (32) reported that mice decomplemented with aggregated human γG-globulin showed normal clearance of carbon particles and decreased clearance of erythrocytes and bacteria coated with specific antibodies. These investigators did not define the class of antibodies utilized in their studies.

The second and third components in the exponential disappearance of immune complexes, prepared with intact antibodies, were thought to represent equilibration and catabolism of the complexes and 6.6S antibodies that survived the phase of rapid removal. During the second phase no preferential removal of complexes was observed and they persisted during the last exponential phase. The second phase showed considerable variation in the half-life, ranging from 1.45 to 6.48 hr.. The reasons for this were not apparent. The third phase lasted a considerable period of time. The mechanism for removal of these complexes is not known. Furthermore, the mechanism of catabolism of γG-globulin has not been elucidated (33). The third phase of removal of immune complexes was closer to removal of antigen alone than to removal of antibodies (γG-globulin) alone. This was best demonstrated in the HSA–anti-HSA system where the mean half-life for the third component was 47 hr (range 40–65 hr), the half-life for HSA was 57 hr, and for anti-HSA alone 76 hr. The half-life of γG-globulin has been shown to vary with the serum concentration of this protein; with increased serum levels the half-life shortens and with decreased levels the half-life increases (33). The γ-globulin level of several rabbits with varying third phase of clearance of immune complexes was measured by cellulose acetate electrophoresis, but no significant variations were detected. Therefore, the variations in removal of the $AgAb_2$

or Ag_2Ab complexes could not have been caused by differences in the γG-globulin concentration.

With reduction and alkylation of interchain disulfide bonds of antibodies, the disappearance of immune complexes from the circulation in the λ–anti-λ, HSA–anti-HSA, and γG–anti-γG systems became a two component exponential process. The half-life of the first component ranged from 1.0 to 2.2 hr and was thought to represent intra- and extravascular equilibration since there was no preferential loss of heavier complexes as determined by density gradient ultracentrifugation. The second exponential component was thought to represent the catabolic phase, which was slightly longer in the HSA–anti-HSA and λ–anti-λ systems than the comparable third component for the intact antibodies. Some rearrangement of immune complexes upon injection into animals may well have taken place by virtue of dilution by the intravascular volume. This was suggested by density gradients that showed some shift of antibodies from the larger complexes to the $AgAb_2$ complexes and 6.6S peak when solutions of complexes were compared to the initial serum specimens of rabbits which received these complexes.

In the γM–anti-γM system with reduced and alkylated antibodies, the rapid phase of removal of $>$25S complexes persisted. The reasons for this were not clear. Preparations of human γM-globulin frequently contain or develop aggregates (34). The preparations of the γM-globulin used in these experiments contained 25S and 29S material. When this preparation was injected into a rabbit, the percentage of counts that corresponded to the percentage of aggregates was quickly removed; the remaining material had the expected phases of equilibration and catabolism. The presence of aggregated γM-globulin could have resulted in rapid clearance of some immune complexes prepared with reduced and alkylated antibodies.

Even though all the purified antibodies had been isolated by virtue of their specificity to antigens coupled to the solid-phase immunoadsorbents, all immune complex preparations in antigen excess contained free antibodies as determined by sucrose density gradient ultracentrifugation. This could either be due to denaturation of antibody molecules or to dissociation of low affinity antibodies during the zone centrifugation. If denaturation had occurred and the binding site alone was altered, the antibodies should have been catabolized as γG-globulins and a fourth exponential component should have become apparent in computer analysis of the data; this, however, was not observed.

Several observations from these studies have relevance to the study of human immune complex diseases. However, caution should be exercised in drawing parallels between primate and rabbit experiments in regard to circulating immune complexes because immune adherence of antigen–antibody–complement complexes to erythrocytes does not occur in rabbits but takes place in man and other primates (35). If our studies are applicable to man, then the soluble im-

mune complexes composed of more than two γG-globulin molecules as antibodies are rapidly cleared from circulation, and this in turn suggests several points. First, the rapid clearance of some immune complexes could account for the difficulty that has been encountered in the demonstration of immune complexes in the sera of patients with systemic lupus erythematosus and other diseases. Secondly, at least some lf the variability in the manifestations of systemic lupus erythematosus may depend on the complexity of antigen–antibody complexes produced at any one time. Thirdly, the low levels of complement seen in these patients should not alter the removal of circulating complexes. If complement were important in the elimination of circulating immune complexes, then markedly low complement levels would cause accumulation of such materials and more injury to several organs.

SUMMARY

Solid phase immunoadsorbents were prepared by coupling antigens to agarose. With this technique specific antibodies were easily isolated in large amounts. The γG-globulin class of antibodies isolated in this manner were not denatured as judged by their normal biological half-life in rabbits. Soluble immune complexes at fivefold antigen excess were prepared from isolated specific antibodies and HSA, human λ-chains, human λG-globulins, and a Waldenström's macroglobulin as antigens. In all these preparations a characteristic immune complex was encountered that represented the smallest stable antigen–antibody union. In the HSA–anti-HSA system they were found to be $AgAb_2$ complexes, and Ag_2Ab complexes in the γG–anti-γG system. These stable complexes fixed complement ineffectively. Also, a spectrum of larger complexes was present in each system, and these complexes fixed complement effectively.

With intact antibodies the disappearance curves of immune complexes from the circulation were composed of three exponential components. The immune complexes larger than $AgAb_2$ were quickly removed from the circulation with half-lives of 0.09–0.37 hr. Their clearance was not dependent on complement components, in that depletion of complement by cobra venom factor and aggregated γG-globulin did not alter the pattern of their removal from the circulation. However, when the interchain disulfide bonds of antibodies were reduced and alkylated, the removal of the λ–anti-λ, HSA–anti-HSA, and γG–anti-γG complexes was altered. In these experiments the disappearance curves were composed of two exponential components and the rapid removal of the greater than $AgAb_2$ complexes did not occur. The immune complexes prepared from reduced and alkylated antibodies fixed complement ineffectively.

The presented data indicate that the rapid removal of circulating immune complexes, containing γG-globulin molecules as antibodies, depends primarily on the number of antibodies involved. Furthermore, complement fixation is not involved in the rapid removal of such complexes. Nevertheless, the rapid re-

moval of immune complexes and their ability to fix complement have similarities for optimal function in that both processes require intact interchain disulfide bonds of antibodies and complexes that exceed the $AgAb_2$ combination.

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