

Effect of reduction and alkylation on allergen-combining properties of reaginic antibody

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The γA -globulin fraction of a reaginic serum was reduced in 0.1M mercaptoethanol followed by alkylation with iodoacetate. Reaginic antibody in the preparation was inactivated by the treatment. The reduced-alkylated γA -globulin did not manifest blocking antibody activity in the Prausnitz-Küstner reaction when it was incubated with allergen. Pretreatment of allergen-coated polyaminostyrene with untreated reaginic γA -globulin decreased the activity of the immune adsorbent to combine with the reagin, whereas treatment with the reduced-alkylated sample did not, indicating that the allergen-combining activity of the reagin was greatly diminished by the reduction-alkylation treatment. The reduced-alkylated γA -globulin from either normal or reaginic serum partially blocked passive sensitization of normal human skin with reagin, suggesting that affinity of reaginic antibody for tissues remained after the treatment. The results definitely indicate that the allergen-combining site(s) in reaginic antibody in the γA -globulin fraction is degraded by the reduction-alkylation treatment.

In previous studies,^{1, 2} skin-sensitizing antibody was detected in the γA -globulin fractions of atopic sera. The reaginic activity in these fractions diminished following reduction of the protein in 0.1M mercaptoethanol and disappeared after reduction followed by alkylation with iodoacetate. These findings are in agreement with the observation of Leddy and associates³ and Gyenes and colleagues⁴ that the reaginic activity in atopic patients' sera was greatly diminished by reduction or reduction and alkylation. Since the sensitization of human skin with reagin probably involves "fixation" of the antibody to tissue constituents, the question remains, whether the inactivation of reaginic activity

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by the reduction-alkylation treatment is due to degradation of either the allergen-combining sites or the "tissue fixation" sites in the antibody molecules. The present investigation was undertaken to study this problem by means of the γ A-globulin fraction of a reaginic serum. The results indicate that it is the allergen-combining site(s) in reaginic antibody to ragweed pollen which is susceptible to the reduction-alkylation treatment.

MATERIALS AND METHODS

Antigen and antibody

A reaginic serum was obtained from a ragweed-sensitive boy suffering from asthma. Blood was taken every month for 6 months, and the serum samples were stored in a frozen state at -40° C. and pooled before use. The serum pool gave a Prausnitz-Küstner (P.K.) reaction with 1:1,000 dilution. A commercial ragweed test allergen* was used to induce skin response.

Gamma A globulin

Gamma A globulin was purified from fresh normal human serum and the reaginic serum by the method described by Vaerman and associates.⁵ The sera were dialyzed against 0.02M phosphate buffer, pH 6.0, to remove euglobulin. Gamma G globulin in the supernatants was removed by precipitation with 0.05M ZnSO_4 at pH 6.8. An equivalent amount of Na_3EDTA was added to the supernatants, dialyzed against distilled water, and concentrated by negative pressure filtration. Gamma A globulin was precipitated by ammonium sulfate

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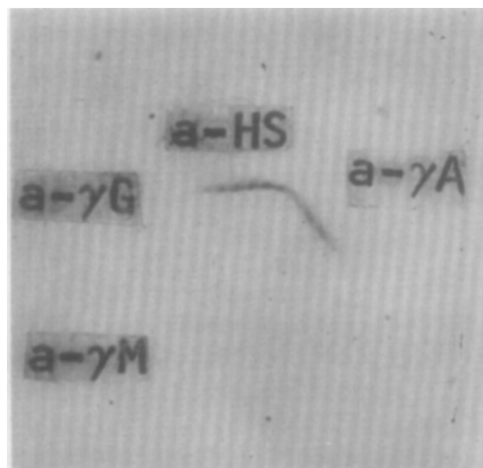


Fig. 1.

Ouchterlony analyses of γ A-globulin fraction of a reaginic serum. A center well was filled with the γ A-globulin preparation. Peripheral wells were filled with antisera specific for γ G ($a\text{-}\gamma\text{G}$), γ A ($a\text{-}\gamma\text{A}$), or γ M ($a\text{-}\gamma\text{M}$) globulin and antihuman serum ($a\text{-HS}$).

at one-half saturation and further fractionated by Pevicon* block electrophoresis. Eluates from segments were tested by immunodiffusion (see below), and those containing only γ A-globulin were pooled.

Reduction and alkylation

Gamma A globulin (0.25 to 0.3 per cent) was reduced in 0.1M mercapto-ethanol at pH 8.0.⁶ After 4 hours at room temperature under N₂, a part of the protein was dialyzed against saline and the remainder was dialyzed against 0.02M iodoacetate for 24 hours, followed by dialysis for 48 hours against borate-buffered saline.

Gel filtration

Gel filtration of γ A-globulin was carried out by means of Sephadex G-200.† One and one half milliliter volumes of a sample were applied to a Sephadex column (1 by 100 cm.) and eluted with borate buffered saline (pH 8.0). Fractions of 1.5 ml. were collected and absorption at 280 m μ was determined.

Immunodiffusion

Double diffusion in agar plates was carried out by the method of Ouchterlony.⁷ The antisera used in the tests‡ were goat antisera against whole human serum and those specific to human 7S γ (γ G) globulin, β_{2M} (γ M) globulin, and to β_{2A} (γ A) globulin. The precipitin bands were stained with azocarmine.

Polyaminostyrene-ragweed complexes

Ragweed allergen-coated polyaminostyrene was prepared by the method described by Richter and associates.⁸ Eighty milligrams of polyaminostyrene§ were suspended in 8.0 ml. of 20,000 PNU per milliliter of ragweed pollen extract and incubated for 30 minutes at room temperature with constant stirring at pH 7.4. The mixture was centrifuged, and the precipitate was washed three times with phosphate-buffered saline, pH 7.4. The complexes were subsequently incubated with normal human serum for one hour at room temperature and washed thoroughly.

Prausnitz-Küstner reactions

The skin reactions were carried out on normal individuals who gave negative skin reactions to ragweed allergen (500 PNU per milliliter). One-twentieth milliliter of an appropriate dilution of γ A-globulin fraction from allergic serum was injected intracutaneously. Unless otherwise described, the sites received 0.02 ml. of ragweed allergen containing 10 PNU 24 hours later. Erythema-wheal reactions, which appeared 15 to 25 minutes after the injections of allergen, were traced on a transparent plate which was applied in the area.

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†Pharmacia, Uppsala, Sweden.

‡From Hyland Labs., Los Angeles, Calif.

§Norsk Hydroelectric Co., Oslo, Norway.

EXPERIMENTAL RESULTS

A γ A-globulin fraction was obtained from normal and reaginic sera by the method of Vaerman and associates⁵ and was tested for purity by immunodiffusion. Both γ A-globulin preparations gave single precipitin bands with identical specificity when tested with anti- γ A-globulin and antihuman serum but no bands with anti- γ G- or anti- γ M-globulin (Fig. 1). The γ A-globulin from the reaginic serum gave P.K. reactions in normal individuals; as little as 0.15 μ g N of the protein produced a definite reaction. A preparation containing 0.6 mg.N was applied to a Sephadex G-200 column and 1.5 ml. fractions were collected. Dilutions of each fraction were injected intracutaneously into normal individuals and the sites were challenged by 2 PNU of ragweed allergen 24 hours later. The distribution of reaginic antibody in the fractions almost paralleled the protein concentration (Fig. 2). The γ A-globulin fraction containing reagin was reduced in 0.1M mercaptoethanol for 4 hours at room temperature. A part of the reduced protein was dialyzed against saline and another part was dialyzed against 0.02M iodoacetate for alkylation. The skin-sensitizing activity of the treated protein was tested by P.K. reactions. The sites which received 12 μ g N of reduced material (without alkylation) gave a slight wheal and erythema which was smaller than that induced with 0.3 μ g N of untreated materials, and the site which received 12 μ g N of reduced-alkylated preparation did not show any reaction (Fig. 3). It is evident that the skin-sensitizing activity of the reaginic antibody was greatly diminished by reduction and disappeared after reduction and alkylation.

The allergen-combining activity of the untreated and reduced-alkylated re-

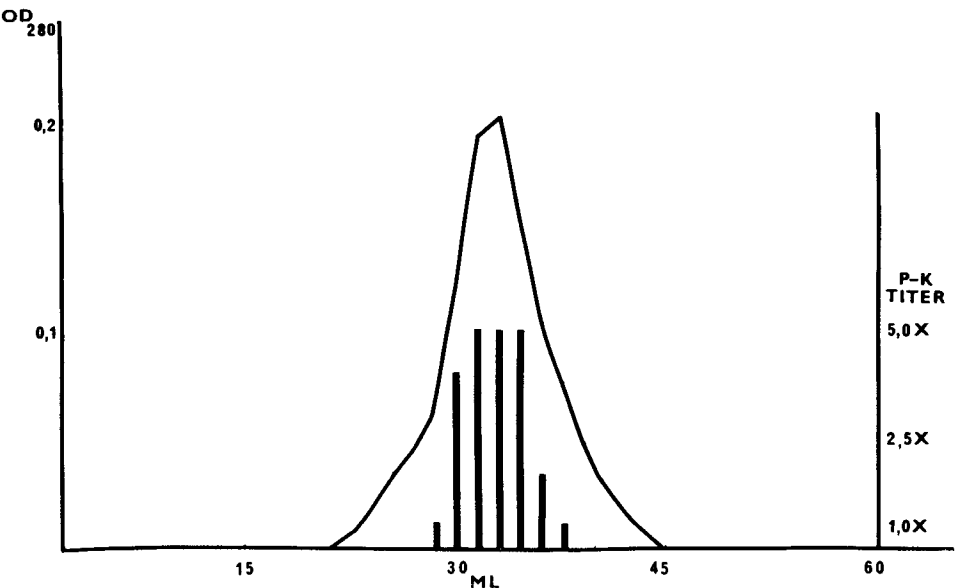


Fig. 2.
Gel filtration of γ A globulin fraction of a reaginic serum.

agin was studied by the method described by Sehon and associates.⁹ As a preliminary experiment, the neutralizing capacity of reagin in the untreated γ A-globulin was determined. Two-tenths milliliter volumes of serial dilutions of the untreated γ A preparation (25 to 100 μ g N per milliliter) and an equal volume of twofold dilutions of ragweed allergen were mixed. After incubation at 3° C. for 24 hours, 0.2 ml. of saline was added to each tube and 0.1 ml. of the mixtures was injected intracutaneously into normal individuals (Table I). The skin sites were challenged with 10 PNU of allergen. As shown in Table I, 160 PNU of allergen was enough to neutralize reagin in 100 μ g N per milliliter of the

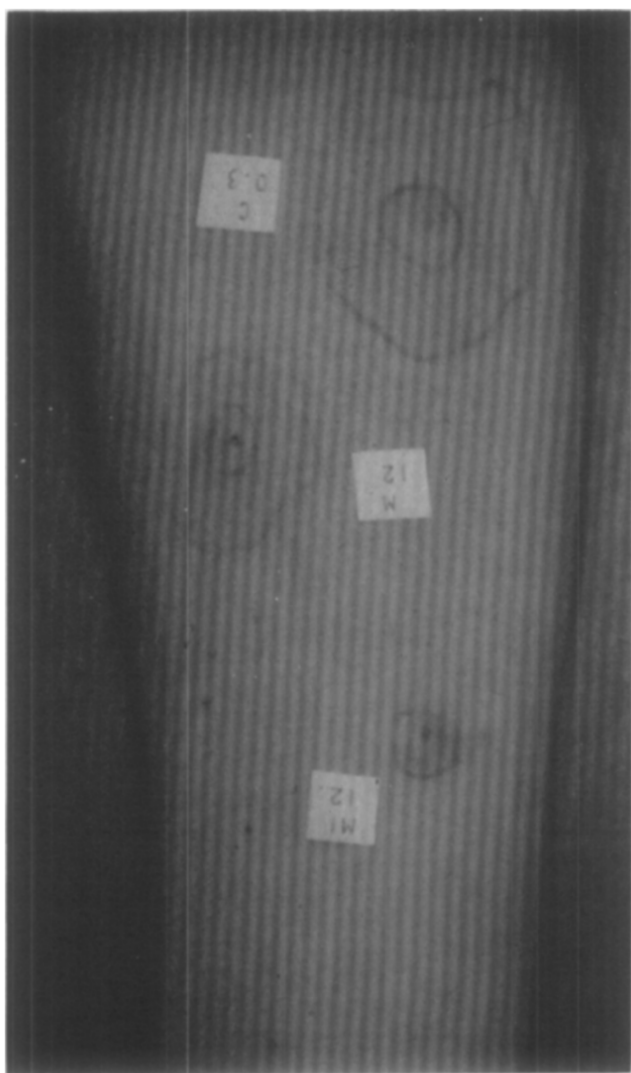


Fig. 3.

P.K. reactions with γ A-globulin fraction of a reaginic serum. The skin sites (C), (M), and (MI) received 0.3 μ gN of untreated sample, 12 μ gN of mercaptoethanol-treated γ A, and 12 μ gN of mercaptoethanol-iodoacetate treated γ A-globulin preparations, respectively.

Table I. Allergen-combining activity of reaginic γA -globulin before and after reduction-alkylation*

Reaginic γA		Normal γA re- duced- alkylated	Allergen							
Un- treated (μg N/ ml.)	Reduced- alky- lated (μg N/ ml.)		0 PNU /ml.	5 PNU /ml.	10 PNU /ml.	20 PNU /ml.	40 PNU /ml.	80 PNU /ml.	160 PNU /ml.	120 PNU /ml.
12.5	0	0	+++	+	±	—	—	—	—	—
25	0	0	++++	+++	++	+	±	—	—	—
100	0	0	++++	+++	+++	+++	++	+	±	—
25	100	0	+++	++	++	+	±	—	—	—
25	0	100	+++	++	++	+	±	—	—	—

*Mixtures of allergen and γA -globulin were injected intracutaneously for passive sensitization. Skin sites were challenged with 10 PNU of allergen 24 hours later.

Table II. Effect of pretreatment of immune adsorbent on its capacity to absorb reagin*

Pretreatment† of immune adsorbent with		Second incubation with reaginic γA ‡			
Agent	μg N/ml.	25 μg N/ml.	12.5 μg N /ml.	6.2 μg N/ml.	3.1 μg N/ml.
Untreated reaginic γA	50		30×41(10)	20×25(7)	7×10(0)
	25§		31×38(10)	19×33(7)	7×6(0)
	12.5		32×39(10)	21×31(8)	9×10(0)
Reduced-alkylated reaginic γA	100	22×30(7)	17×19(6)	6×7(0)	—
Reduced-alkylated normal γA	100	29×34(9)	15×17(6)	8×8(0)	—
Saline	0	25×34(7)	16×19(6)	6×7(0)	—
No immune adsorbent		46×45(12)	29×44(11)	20×26(7)	11×15(5)

*Immune adsorbents were pretreated with γA -globulin and then incubated with reaginic γA . Reaginic activity in the final supernatant was tested by P.K. reaction. Numerals in the table represent diameters of erythema. Average diameter of wheal is shown in parentheses in millimeters.

†Two volumes of a γA preparation were incubated with 1 volume of a suspension of ragweed-coated polyaminostyrene (4 mg. per milliliter) for 1 hour at room temperature.

‡After the second incubation for 1 hour at room temperature, 0.05 ml. supernatant was injected for passive sensitization and the sites were challenged with 0.02 ml. of 500 PNU per milliliter ragweed pollen extract 24 hours later.

§First supernatant (0.05 ml.) gave a P.K. reaction 20×22(6).

γA -globulin. The neutralization test of reagin with allergen was repeated in vivo. One-tenth milliliter volumes of 33 μg N per milliliter of the γA -globulin were injected into 6 sites of normal individuals. Twenty-four hours later, these sites received 0.1 ml. of twofold dilutions of allergen containing 13 to 213 PNU per milliliter. Strong erythema-wheal reactions were observed at all sites. After 24 hours, when the skin reactions had completely disappeared, the sites were challenged with 10 PNU of ragweed allergen. The sites receiving 27 PNU per milliliter of allergen for the second injection showed slight erythema-wheal reactions, whereas the sites injected with twice as much allergen did not. These

results indicate that the reagin was neutralized with the same amount of allergen, irrespective of whether the reagin and allergen were mixed in vitro or allergen was injected into the sites 24 hours after the passive sensitization with reagin.

The allergen-combining properties of the reduced-alkylated γ A-globulin were then tested. Two-tenths milliliter of the reduced-alkylated γ A-globulin (100 μ g N per milliliter) and equal volumes of serial dilutions of allergen (5 to 80 PNU per milliliter) were incubated for 24 hours at 3° C. As controls, reduced-alkylated normal γ A-globulin (100 μ g N per milliliter) and allergen were incubated. Two-tenths milliliter of the untreated reaginic γ A-globulin (25 μ g N per milliliter) was then added to each mixture, incubated for 1 hour at room temperature, and 0.1 ml. of the mixtures was injected into normal individuals (Table I). Twenty-four hours later, these sites were challenged with 10 PNU per milliliter of allergen. The skin sites sensitized with mixtures containing reduced-alkylated reagin and those containing similarly treated normal γ A-globulin gave comparable reactions (Table I) indicating that the reduced-alkylated reagin did not combine with allergen.

It was reported by Gyenes and associates¹⁰ and Delorme and others¹¹ that allergen-coated polyaminostyrene absorbs reagin in vitro. The latter authors also found that the treatment of the immune adsorbent with reaginic serum resulted in a decrease in the capacity of the adsorbent to combine with reagin. In view of these findings, the allergen-combining properties of reduced-alkylated γ A reaginic antibody were studied by means of the immune adsorbent. As a preliminary experiment, twofold dilutions of a suspension of ragweed allergen-coated polyaminostyrene were incubated with the reaginic γ A-globulin for 1 hour at room temperature. After centrifugation, the presence of reagin in the supernatants was tested by a P.K. reaction. It was found that 2 to 4 mg. of the immune adsorbent was enough to absorb most reaginic activity in 25 μ g N of the γ A-globulin fraction. Thus, 2 mg. aliquots of the immune adsorbent were suspended in 1 ml. volumes of serial dilutions of the reaginic γ A (12.5 to 50 μ g N per milliliter) or 100 μ g N per milliliter of the reduced-alkylated γ A-globulin from reaginic or normal serum. The suspensions were incubated for one hour at room temperature with occasional mixing, and centrifuged. The precipitates were washed three times with saline and again incubated for one hour with reaginic γ A-globulin containing 25 μ g N. They were centrifuged and twofold dilutions of the supernatants were injected intracutaneously for passive sensitization. The results of the skin reactions are shown in Table II. As reported by Delorme and associates,¹¹ the pretreatment of the immune adsorbent with reaginic γ A decreased the capacity of the adsorbent to combine with the reagin, whereas the treatment with the reduced-alkylated γ A-globulin did not have any detectable effect on the reagin-combining capacity of the adsorbent.

Since Delorme and others¹¹ reported that two consecutive incubations of the immune adsorbent with the reaginic serum were more effective for the saturation of the adsorbent, the experiments were repeated with a different procedure. Two of the six identical preparations of the immune adsorbent (2 mg.) were suspended in 0.5 ml. of 200 μ g N per milliliter of untreated γ A, with 400 μ g N per milliliter of reduced-alkylated γ A or saline as controls. After incubation

for one hour at room temperature, the tubes were centrifuged and the precipitates were reincubated in the similar preparations. The final precipitates of immune adsorbent were washed three times with saline, and two aliquots of similarly treated adsorbents were resuspended in 0.5 ml. of reagenic γ A containing 10 μ g N or 5 μ g N. After incubation for one hour, the supernatants were tested for the presence of reagin. The results, shown in Table III, indicated that the reduced-alkylated γ A-globulin did not inhibit the absorption of the reagin to the immune adsorbent.

In a previous paper,¹² it was found that pretreatment of skin sites with normal γ A-globulin blocked passive sensitization of the sites with reagenic antibody, indicating that γ A-globulin has affinity for the skin sites with which reagin combines upon sensitization. In order to study the effect of reduction and alkylation on the affinity of γ A reagenic antibody for the tissue sites, the blocking effect of reduced-alkylated γ A-globulin was tested. Five micrograms nitrogen of the reduced-alkylated reagenic γ A-globulin, untreated or treated normal γ A-globulin in 0.1 ml. was injected intracutaneously into normal individuals. After 24 hours, these sites and a control site received 0.05 ml. of reagenic serum (1:500) followed by ragweed allergen (2 PNU) 24 hours later. The control site induced an erythema of 30 by 40 mm. with a definite whealing (11 mm.) whereas the sites receiving the untreated normal γ A-globulin did not. Partial inhibition of P.K. sensitization was obtained with reduced-alkylated γ A-globulin. The sites which received either normal or reagenic reduced-alkylated γ A gave a slight erythema of 15 to 20 mm. diameter, but whealing was less than 5 mm. These findings indicate that some affinity of γ A-globulin for skin sites remained after reduction and alkylation of the protein.

DISCUSSION

It was confirmed that the skin-sensitizing antibody in γ A-globulin fraction of a reagenic serum was inactivated by reduction in 0.1M mercaptoethanol followed by alkylation. The reduced-alkylated antibody did not have the properties of a blocking antibody, indicating that the treated antibody did not combine with allergen either in vivo or in vitro. The possibility remains that tissue cells

Table III. *Effect on reagin uptake of immune adsorbent pretreated with reduced-alkylated reagenic antibody**

Pretreatment† of immune adsorbent with		Third incubation of immune adsorbent with reagenic γ A (μ g N/ml.)	
Agent	μ g N/ml.	20	10
Untreated reagenic γ A	200	29 (10)	27 (8.5)
Reduced-alkylated reagenic γ A	400	18 (3)	0
Saline	0	17 (3)	0

*Numerals in this table represent average diameters in millimeters of erythema and wheal (in parentheses) of P.K. reaction with the final supernatant.

†Two consecutive incubations of immune adsorbent with γ A-globulin.

may play an intimate part in the reagin-allergen interaction and, therefore, that the lack of allergen-neutralizing capacity of the reduced-alkylated antibody may be due to the loss of affinity of the treated antibody for skin tissues. However, this possibility is unlikely, since it has been demonstrated that participation of tissue cells is not essential for reagin-allergen combination: Richter and associates¹³ have demonstrated that reagin combines with allergen in vivo without inducing erythema-wheal reaction when these reagents were introduced into the same skin site, successively, and Gyenes and Sehon¹⁰ as well as Delorme and colleagues¹¹ reported that reaginic antibody is absorbed to allergen-coated immune adsorbents. Our recent studies also provided evidence that ragweed allergen and skin-sensitizing antibody combine in vitro and the resulting complexes do not dissociate by gel filtration.¹⁴ The present data indicated that treatment of allergen-coated polyaminostyrene with reaginic antibody, but not with reduced-alkylated antibody, reduced the capacity of the immune adsorbent further to absorb the antibody. It is evident that the allergen-combining capacity of skin-sensitizing antibody is lost or greatly diminished by the reduction and alkylation treatment. It was also found that reduced-alkylated γ A-globulin from reaginic serum and normal serum partially blocked passive sensitization of normal skin with reagin, indicating that affinity of γ A-globulin for skin tissues remained after the treatment. These observations are in agreement with the previous findings¹⁵ that the heavy chain fraction of normal γ A-globulin, which was obtained after reduction and alkylation of the protein, has the blocking activity; and indicate that the inactivation of reaginic antibody by reduction-alkylation treatment is mainly due to degradation of allergen-combining sites in the antibody molecules.

The effect of reduction in mercaptoethanol on antibody activity has been studied by many investigators. Grubb and Swahn,¹⁶ as well as Fudenberg and co-workers,¹⁷ reported that hemagglutinating activity of γ M antibody but not γ G antibody was lost by the treatment. With pure γ M antihapten rabbit antibody, however, Onoue and associates¹⁸ recently demonstrated that the antibody-combining sites remained after reduction and alkylation. Schrohenloher and associates,¹⁹ who dissociated rheumatoid factor to 7S subunits by reduction with ethylmercaptan, or with mercaptoethanol, showed that the 7S fragment is capable of combining with γ globulin. With respect to γ A-globulin antibody, Rawson and Abelson²⁰ found that hemagglutinating activity of γ A isoagglutinin was not significantly diminished by reduction and alkylation. In our experiment,²¹ however, a decrease of hemagglutinating activity of anti-A isoagglutinin of γ A-globulin type was demonstrated. Furthermore, our recent studies on the isoagglutinin provided evidence that stability of antigen-combining sites in antibody molecules is different, depending on the classes of immunoglobulin with which the antibodies are associated.²² After reduction in 0.1M mercaptoethanol for 2 hours, and alkylation with iodoacetate, the concentration of both polymer and monomer types of γ A-globulin antibody decreased to 20 to 25 per cent, whereas 90 per cent of the γ G-antibody activity remained. The γ M-globulin antibody, which can combine with type A erythrocytes, decreased to 2.4 per cent of the original concentration. These findings indicate that degradation of anti-

gen-combining sites by the reduction-alkylation treatment is not characteristic for γ M-globulin antibody and that the combining sites in γ A-globulin antibodies, as well, are partially degraded by the treatment. The present data on the effect of reduction and alkylation on reaginic antibody neither support nor exclude the idea that reaginic antibody is γ A-globulin.

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