THE ROLE OF DISULFIDE BONDS IN THE COMPLEMENT-FIXING AND PRECIPITATING PROPERTIES OF 7S RABBIT AND SHEEP ANTIBODIES

By PETER H. SCHUR,* M.D., AND GARY D. CHRISTIAN, PH.D.

(From the Department of Immunochemistry and Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D. C.)

(Received for publication, April 30, 1964)

The functional structure of gamma globulins and antibodies has recently come under intensive investigation through the use of reduction and alkylation of disulfide bonds (1). This method has revealed the great similarity in fundamental polypeptide chain structure among many species as well as between the different classes of immunoglobulins (2). By contrast, reduction of disulfide bonds affects immunological properties of different antibodies to varying degrees.

The greater number of 19S antibodies,—*viz.* cold agglutinins, heterophile antibodies, typhoid O agglutinins,—lose serological activity after treatment with a 0.1 M concentration of mercaptan (3–10). Most 7S antibodies are unaffected by this same concentration of mercaptan (6, 7, 11, 12).

However, more recently a number of reports have appeared indicating that a number of 7S antibodies of different species can lose immunological activity after 0.1 M mercaptan treatment (11, 13). Of particular interest is a report by Wiedermann *et al.* (14) who showed that 5 human sera with antitissue (7S) antibodies lost their complement-fixing activity after treatment with 0.1 M mercaptoethanol, without losing their ability to react with antigen. When the 7S gamma globulin was isolated from untreated sera and heat-aggregated it reacted with rheumatoid factor and bound complement. After treatment with 0.1 M mercaptoethanol the ability to react with rheumatoid factor was unaffected, while these preparations no longer bound a significant amount of complement. However, the full significance of this decrease in complement-fixing ability cannot be evaluated because of the limitations of their method of complement fixation.

In a previous publication, investigating the complement-fixing properties of 7S gamma globulin and its 5S fragment, it was observed that treatment with as little as 0.01 M cysteine could decrease the complement-fixing efficiency of preformed immune aggregates containing 7S rabbit gamma globulin (15).

This investigation was undertaken to quantitatively study the relationship

---

* Present address: The Rockefeller Institute.

of complement-fixing and precipitating ability of 7S rabbit and sheep antibodies to the number of disulfide bonds in the molecules.

Materials and Methods

Antibodies.—7S rabbit (7S RGG) and 7S sheep antibodies (7S SGG) to human serum albumin (HuSA) were isolated and checked for purity by methods previously described (15).

Protein, Antibody, and Antigen Nitrogen (N).—These were determined by the Markham microKjeldahl method (16, p. 476). Protein values were determined assuming 6.25 mg protein for each mg N (16). Antibody N was determined by the precipitin technique (described in reference 16, p. 22). The precipitating ability, *i.e.*

$$\frac{\dfrac{\text{mg antibody N/ml}}{\text{mg protein/ml}} \text{ for treated antibody}}{\dfrac{\text{mg antibody N/ml}}{\text{mg protein/ml}} \text{ for untreated antibody}} \times 100 \text{ per cent,}$$

of treated antibody was plotted against the concentration of 2-mercaptoethanol used, on semilog paper.

Reduction and Alkylation.—2-Mercaptoethanol (2-ME) was added to 1 to 3 per cent solutions of gamma globulin in phosphate-saline buffer (pH 6.9, 0.17 M), and stirred continuously, in a closed vessel, for 2 hours at room temperature. The reduced solutions were then dialyzed against a large excess of 0.02 M iodoacetamide (IOD) in the phosphate-saline buffer, for 20 hours at 4°C. The solutions then were extensively dialyzed against the phosphate-saline buffer. The reduction procedure often resulted in denaturation of some material, especially when a high concentration of 2-ME was used. These denatured products were removed by high speed centrifugation prior to study. Solutions were concentrated by ultrafiltration, if necessary, prior to testing for precipitating ability. Solutions were stored at −20° or 4°C for a short time just prior to use. The antibody N of each treated 7S RGG and 7S SGG preparation was determined.

Complement Fixation Using Guinea Pig Serum.—Complement fixation was performed as previously described, using washed, preformed antibody-antigen (immune) aggregates formed at equivalence (15). This method gives results which vary in their $C.F._{50}$[1] by ± 10 per cent. The per cent of complement ($C'H_{50}$) fixed was plotted against the concentration of antibody N, on semilog paper. The relative complement-fixing efficiency of different preparations of treated and untreated 7S antibodies to fix complement were quantitatively compared:

$$\frac{C.F._{50} \text{ for treated antibody}}{C.F._{50} \text{ for untreated antibody}} \times 100 \text{ per cent.}$$

These complement-fixing efficiencies were plotted against the concentration of 2-ME used, on semilog paper. This method is more informative than a comparison of the complement-fixing abilities of different preparations at the same protein concentration. Preparations were also compared as regards their maximal complement-fixing ability.

Disulfide Bonds.—The number of total and interpolypeptide chain disulfide bonds[2] per mole of gamma globulin was determined by a modification (18) of the amperometric titration

---

[1] $C.F._{50}$ is that quantity of antibody N required to fix 50 per cent of the total complement ($C'H_{50}$ units) present.

[2] There are 4 polypeptide chains in 7S rabbit gamma globulin connected by interpolypeptide chain disulfide bonds (17).

methods of Cecil and Wake (19) and Franek and Lankas (20). The number of total disulfide bonds were titrated with mercuric chloride, after the sample had been reduced in an excess of 0.5 M sodium sulfite (under nitrogen) in the presence of 8.0 M guanidine, pH 6.5, at room temperature. The standard deviation of the method was ±0.5 disulfide bonds for unreduced 7S RGG. The number of disulfide bonds left in gamma globulin after treatment with mercaptans was similarly titrated, but only after the sulfhydryl groups reduced by the mercaptan had been alkylated with iodoacetamide and the excess iodoacetamide removed. The interdisulfide bonds were titrated with phenylmercuric hydroxide at 0°C, after reduction with sodium sulfite (under nitrogen) by the method of Cecil and Wake (19). As will be described elsewhere in greater detail (18) the number of apparent interdisulfide bonds depends upon the concentration of sodium sulfite used for reduction. When 0.5 to 0.75 $\times 10^3$ M sulfite/M protein was used, 2 disulfide bonds were titrated for 7S and 5S RGG. When 1.25 to 2.5 $\times 10^3$ M sulfite/M protein was used, 3 disulfide bonds were titrated, for 7S and 5S RGG. However, when 2.75 $\times 10^3$ M sulfite/M protein was used, 5 disulfide bonds were titrated for 5S RGG; and when 3.5 to 5.0 $\times 10^3$ M sulfite/M protein was used, 4 disulfide bonds were titrated for 7S RGG. We have arbitrarily designated the values obtained when 1.25 to 2.5 $\times 10^3$ M sulfite/M protein was used as representing the number of interchain disulfide bonds.

The titration curves for 7S and 5S SGG showed linear relationships between the number of interdisulfide bonds reduced and the amount of sulfite used. This was in contrast to the stepwise increase in number of bonds reduced in RGG. Therefore no attempt was made to designate interdisulfide bonds for SGG.

For these determinations, the molecular weights were assumed to be 150,000 for 7S RGG (21), 106,000 for 5S RGG (21), and 160,000 for 7S SGG (22).

Pepsin Digestion.—This was performed as previously described (15).

Immunoelectrophoresis.—Microimmunoelectrophoresis in agar gel was performed by the method of Scheidegger (23).

Analytical Ultracentrifugation.—The determination of sedimentation constants (s_{20w}) was performed as previously described (15).

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

RESULTS

Immunological Properties.—Immunoelectrophoresis of the 7S RGG and 7S SGG preparations, against anti-whole rabbit and anti-whole sheep serum respectively, showed that they consisted almost entirely of $gamma_2$ globulin, except for an occasional trace of $alpha_2$ globulins (not detected by paper electrophoresis). Immunoelectrophoresis of the 7S RGG and 7S SGG against human serum albumin (HuSA) revealed that their precipitating antibody activity resided only in the slow $gamma_2$ region for the 7S RGG and in the fast $gamma_2$ region for the 7S SGG.

Properties of Treated and Untreated Rabbit Gamma Globulin (7S RGG) (Figs. 1 and 2, Table I).—

No treatment: 7S RGG gave a single peak in the ultracentrifuge with a sedimentation constant, $s_{20,w}$, of 6.4S. Thirteen to 20 per cent of the total protein in different preparations was precipitating antibodies.

Only 1.1 μg antibody N of 7S RGG was needed to fix 50 per cent of complement. Different preparations of 7S RGG did not vary in their $C.F._{50}$ values by more than about ±15 per cent.

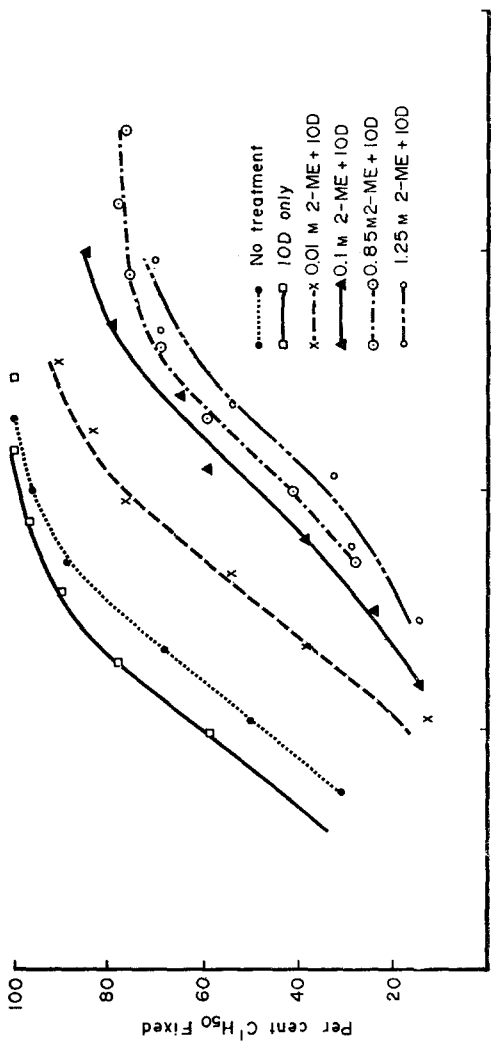

FIG. 1. The complement-fixing abilities of 7S rabbit anti-HuSA, before and after treatment with 2-mercaptoethanol (2-ME) and/or iodoacetamide (IOD).

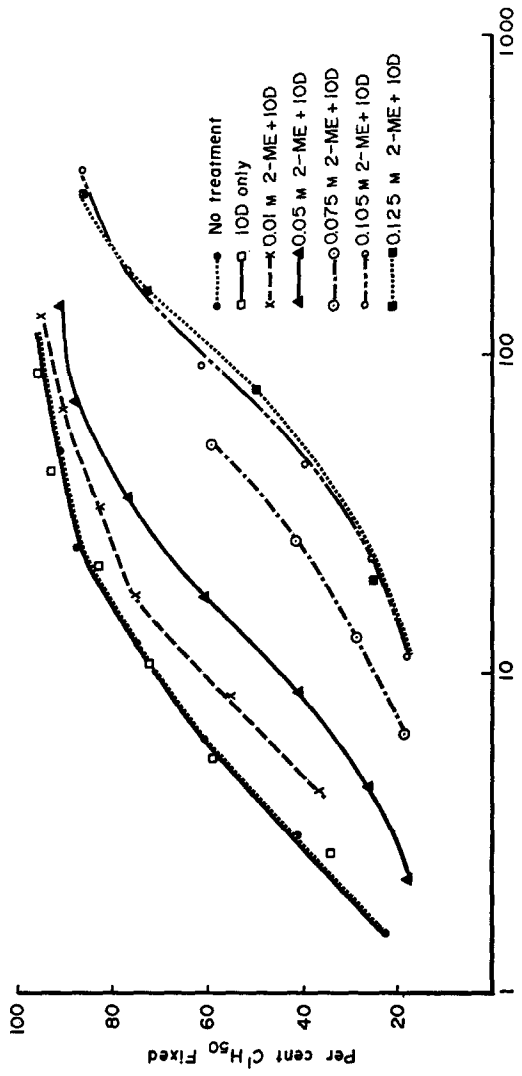

FIG. 2. The complement-fixing abilities of 7S sheep anti-HuSA, before and after treatment with 2-mercaptoethanol (2-ME) and/or iodoacetamide (IOD).

7S RGG had 22.5 ± 0.5 disulfide bonds, of which 3 were interpolypeptide chain disulfide bonds. There were 0 to 0.67 free titratable sulfhydryl groups.

Iodoacetamide treatment: 7S RGG treated with iodoacetamide alone had a sedimentation constant of 6.4S.

TABLE I

The Effect of 2-Mercaptoethanol (2-ME) Treatment on Rabbit and Sheep Antibodies

Preparation	2-ME treatment*	Sedimentation constant		Precipitating ability‡	Complement-fixing efficiency‡	Disulfide bonds§
		per cent	*S*			
7S rabbit gamma globulin	0	100	6.4	100	100	22.5
	Iodoacetamide	100	6.4	100	138	22.7
	0.01 M	100	6.4	97.5	39	20
	0.1 M	100	6.4	98.0	13	15.5
	0.3 M	—	—	96.5	8	—
	0.85 M	75	6.4	85	8	—
		25	3.5			
	1.45	—	—	64	6	10.3
	1.65	70	6.4	34	—	—
		30	3.5			
	>1.65	—	—	0	—	—
7S sheep gamma globulin	0	100	6.4	100	100	22
	Iodoacetamide	100	6.4	100	100	22
	0.01	100	6.4	100	64	20.8
	0.05	100	6.4	100	41	14.2
	0.075	90	6.4	84	12	10.5
		10	3.8			
	0.095	—	—	—	—	—
	0.105	—	—	73	6	—
	0.145	—	—	70	—	10
	>0.145	—	—	0	—	—

* Whenever 2-ME was used, 0.02 M iodoacetamide was used afterwards.

‡ Per cent of activity of untreated preparation.

§ Number of total disulfide bonds in untreated preparations and number of intact disulfide bonds left in 2-ME treated preparations.

Only 0.74 μg antibody N was needed to fix 50 per cent of complement. This was a 38 per cent increase in complement-fixing efficiency. There was no change in precipitating ability.

There were 22.7 titratable disulfide bonds.

0.01 M 2-ME treatment: This protein still had a sedimentation constant of 6.4S, and had retained 97.5 per cent of its precipitating ability. However, there was now a 61 per cent decrease in complement-fixing efficiency, and also a reduction of 2 to 3 disulfide bonds.

0.1 M 2-ME treatment: This preparation gave a single peak in the ultra-

centrifuge with a sedimentation constant of 6.4S. Ninety-eight per cent of the precipitating ability was retained, but there was a 90 per cent decrease in complement-fixing efficiency associated with the reduction of 7.4 disulfide bonds.

0.3 M 2-ME treatment: This preparation retained 96.5 per cent of its precipitating ability, but had lost 92 per cent of its complement-fixing efficiency.

0.85 M 2-ME treatment: This preparation consisted of a mixture of about 75 per cent 6.4S and 25 per cent 3.5S protein. Its precipitating ability had decreased 15 per cent, but its complement-fixing efficiency had decreased no further than 90 per cent.

1.45 M 2-ME treatment: This preparation had lost 36 per cent of its precipitating ability, and 94 per cent of its complement-fixing efficiency. There was also a possible diminution in maximal complement-fixing ability. In addition, 12.2 of the disulfide bonds had been reduced.

1.65 M 2-ME treatment: This preparation consisted of a mixture of about 70 per cent 6.4S and 30 per cent 3.5S protein. It had lost 66 per cent of its precipitating ability.

When higher concentrations of 2-ME were used, the preparations would no longer aggregate with antigen and therefore could not be tested for complement fixation.

Properties of Treated and Untreated Sheep Gamma Globulin (7S SGG) (Figs. 3 and 4, Table I).—

No treatment: 7S SGG had a sedimentation constant of 6.4S. 4.3 μg antibody N would fix 50 per cent of complement. Eighteen to 20 per cent of the total protein in different preparations was precipitating antibody. This protein had 22 disulfide bonds and there were no free titratable sulfhydryl groups.

Iodoacetamide treatment: This preparation had the same properties as untreated 7S SGG.

0.01 M 2-ME treatment: This treatment did not change the sedimentation constant or precipitating ability. There was, however, a 36 per cent decrease in complement-fixing efficiency and a reduction of 1.2 disulfide bonds.

0.05 M 2-ME treatment: This preparation consisted entirely of 6.4S protein. There was no change in precipitating ability. Complement-fixing efficiency was decreased 60 per cent, and 7.8 disulfide bonds were reduced.

0.075 M 2-ME treatment: This preparation, consisting of a mixture of 90 per cent 6.4S and 10 per cent 3.8S protein, had lost 16 per cent of its precipitating ability and 87 per cent of its complement-fixing efficiency. 11.5 disulfide bonds had been reduced.

0.105 M 2-ME treatment: Twenty-seven per cent of its precipitating ability and 94 per cent of its complement-fixing efficiency was lost.

0.145 M 2-ME treatment: Thirty per cent of its precipitating ability was lost. Twelve disulfide bonds had been reduced.

Preparations of 7S SGG treated with 0.095 M or more 2-ME, were unstable,

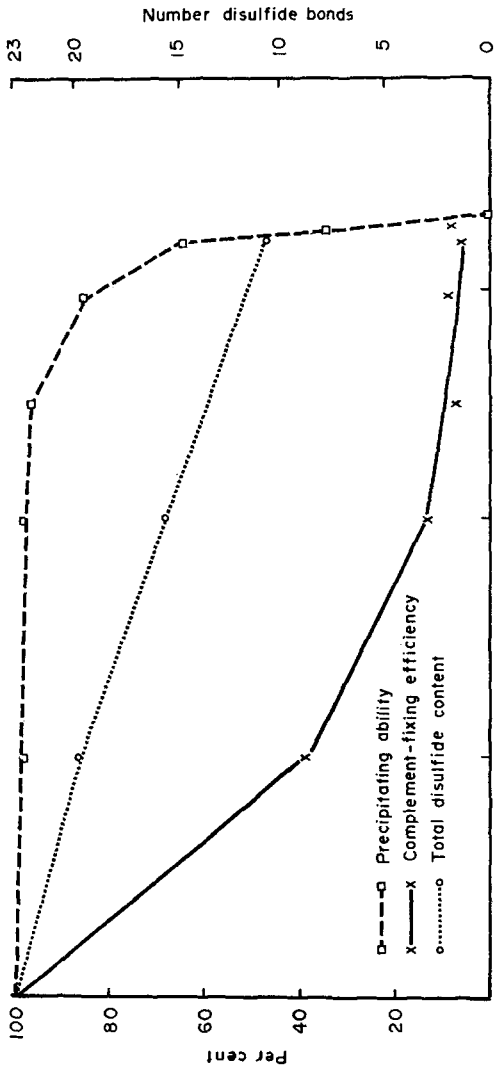

FIG. 3. The effect of 2-mercaptoethanol (2-ME) treatment on 7S rabbit antibody to HuSA: precipitating ability, complement-fixing efficiency, and disulfide content.

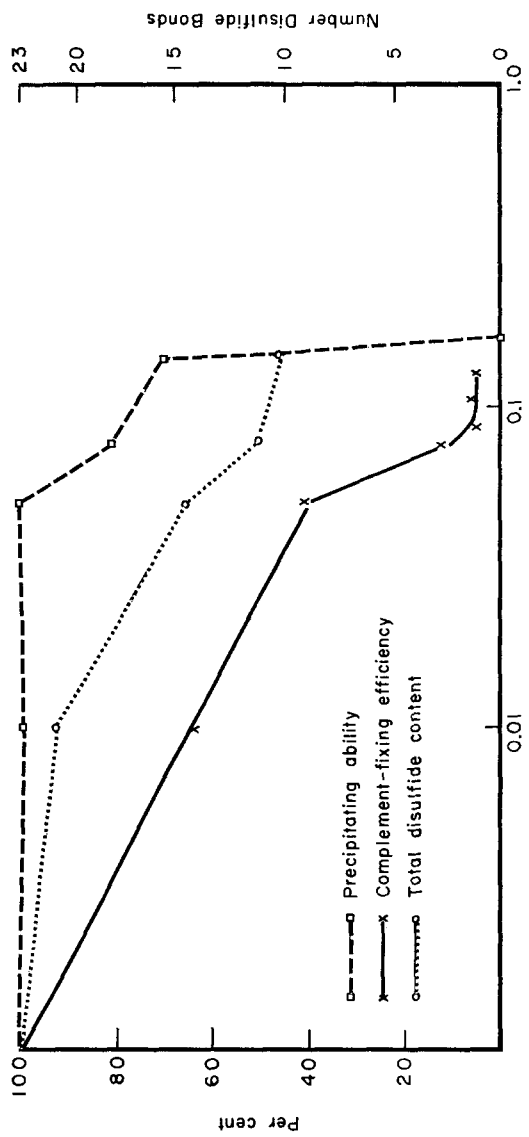

FIG. 4. The effect of 2-mercaptoethanol (2-ME) treatment on 7S sheep antibody to HuSA: precipitating ability, complement-fixing efficiency, and disulfide content.

and split within about a week to 3.8S proteins at 4°C; but only after about a month at −20°C. 7S SGG treated with more than 0.145 M 2-ME, and then aggregated with antigen, would occasionally even dissolve after a few days, due presumably to the formation of these non-aggregating 3.8S fragments.

Treatment of 7S RGG with Pepsin and/or 2-ME.—Digestion of 7S RGG with pepsin yielded a smaller 5S fragment which retained most of the precipitating ability of the intact 7S antibody (15). However, 5S RGG was able to fix no more than 40 per cent of complement (15). 5S RGG had 17 total, and 3 interpolypeptide chain, disulfide bonds. When 5S RGG was treated with 0.01 M 2-ME, one disulfide bond was reduced and the 5S fragment split in half to 3.5S proteins which would not precipitate with antigen (24). These 3.5S proteins would therefore each have 8 disulfide bonds.

When immune aggregates containing 5S RGG were treated with 0.01 M 2-ME they dissolved, and the solution would no longer fix complement (15).

Treatment of 7S RGG first with 0.01 M 2-ME and then iodoacetamide, followed by pepsin digestion, resulted in a preparation consisting of 10 per cent 5S and 90 per cent 3.5S proteins. This latter preparation had 16 disulfide bonds (8 for each 3.5S fragment), and would not aggregate with antigen, just as when 5S RGG was split to 3.5S with 0.01 M 2-ME.

DISCUSSION

A concentration of 0.1 M mercaptan originally was thought to destroy the immunological activities of most, if not all, 19S antibodies, but not to affect 7S antibodies. More recently, as reviewed in the introduction, it has become apparent that the activities of a number of 7S antibodies also can be affected by mercaptans. The decrease in activity has been thought to be due to the reduction of disulfide bonds (11, 25).

We found a total of 23 disulfide bonds in 7S RGG by reduction with 0.5 M sodium sulfite in 8 M guanidine (18). This figure is in good agreement with those determined by other means: 18 to 25 cystine residues per mole of rabbit gamma globulin were found by amino acid analysis (25–28), and 18 to 22 disulfide bonds could be reduced by mercaptans in the presence of urea (17, 29).

The number of interpolypeptide chain disulfide bonds in gamma globulin usually has been defined as the number of disulfide bonds reduced in the absence of a detergent or denaturing agent. Values of four (30), five (17), and seven (29) have been obtained for 7S rabbit gamma globulin. We found that 11 to 12 "interchain" disulfide bonds were reduced in the presence of 2-ME alone. It becomes evident that this method gives inconsistent results for the interchain disulfide bonds. When 7S (and 5S) rabbit gamma globulins were analyzed for interchain disulfide bonds by the method of Cecil and Wake (19), we obtained only 3 disulfide bonds over a relatively wide range of concentrations of reducing agents.

The study of disulfide bonds in gamma globulin also can be approached from a study of their functional role (11, 25). Nisonoff *et al.* (24) have shown that the reduction of "a single labile disulfide bond" in 5S rabbit antibody converted a precipitating antibody into 2 non-precipitating (univalent) fragments. Based on Porter's model for 7S rabbit antibody, this bond is an inter-(polypeptide)-chain disulfide bond[3] (17). Palmer and Nisonoff later showed that this same "critical disulfide bond" also could be reduced in intact 7S antibody (21). We have confirmed these studies, using 0.01 M 2-ME.

However, the treatment of 7S RGG with 0.01 M 2-ME reduced a total of 2 to 3 disulfide bonds, including this 1 interchain disulfide bond [S—$S_{(1)}$]. The other reduced disulfide bond(s) appear to be in that portion of 7S rabbit antibody removed by pepsin digestion; *i.e.*, in Porter fragment III.

Treatment with 0.01 M 2-ME reduced 2 to 3 disulfide bonds in 7S RGG, and 1 disulfide bond in 7S SGG. This reduction was not associated with any apparent fragmentation of the 7S molecules nor any decrease in precipitating ability of the antibodies. However, both preparations were less efficient in fixing complement: 60 per cent for 7S RGG and 36 per cent for 7S SGG.

Seven to 8 disulfide bonds could be reduced in 7S RGG and 7S SGG by treatment with 0.1 and 0.05 M 2-ME, respectively. This reduction also did not affect their precipitating abilities nor cause fragmentation of the 7S molecules. There was, however, yet a further decrease in complement-fixing efficiency: 90 per cent for 7S RGG and somewhat less for 7S SGG, 64 per cent.

The complement-fixing efficiency of 7S RGG could be decreased no further than this 90 per cent, even when more than 7 disulfide bonds were reduced by a higher concentration of mercaptan. However, 7S SGG was different in that 10 to 11 disulfide bonds had to be reduced, by treatment with 0.075 to 0.085 M 2-ME, in order to achieve this same maximal 90 per cent reduction in complement-fixing efficiency.

When more than 10 to 11 disulfide bonds were reduced in 7S RGG and 7S SGG, by treatment with more than 0.3 and 0.075 M 2-ME respectively, there was a reduction in precipitating ability. When 12 disulfide bonds were reduced in 7S RGG and 7S SGG, by approximately 1.5 and 0.15 M 2-ME respectively, 50 per cent of the precipitating abilities of the preparations had been lost. When more 2-ME was used, the preparations would barely, if at all, precipitate with antigen.

Treatment of 7S RGG and 7S SGG with more than 0.85 and 0.075 M 2-ME respectively, also resulted in fragmentation of a portion of the 7S proteins to 3.5S to 3.8S proteins. In a limited number of observations there appeared to be no correlation between the increasing amounts of 2-ME used, or decrease in precipitating ability, with the degree of fragmentation. These 3.5S to 3.8S

[3] This bond will be referred to later in this paper as [S—$S_{(1)}$].

fragments are probably similar to, if not identical with, the 7S halves found by Palmer *et al.* (31) after they acidified 0.1 M mercaptan reduced rabbit gamma globulin. However, they might also represent the A chains of Porter (17).

These results revealed a difference in the ease of reduction of disulfide bonds and effect on complement fixation in two species (rabbit and sheep) of antibodies. A similar study was reported by Wiedermann *et al.* (14) on human antibodies. They showed a decrease in complement-binding ability of heat-aggregated, mercaptoethanol-treated 7S human gamma globulin at the one protein concentration they used.

In the present investigation we have shown that 2-ME treatment caused little if any decrease in maximum complement-fixing ability. This is not unexpected as the complement-fixing property of antibodies is dependent on its inherent ability to aggregate (32). However, this reduction of disulfide bonds caused a decrease in complement-fixing efficiency. It therefore appears that quantitative complement-fixing efficiency is related to the disulfide bond structure of antibodies. In addition, the precipitating ability of antibodies also appears to be related to the disulfide bond structure of antibodies, but in a different manner from complement-fixing efficiency.

The disulfide bonds which are more labile to mercaptan reduction appear to be associated with complement fixation, while the disulfide bonds which are more resistant to mercaptan reduction appear to be associated with precipitating ability. These data fail, however, to fully explain whether the decrease in complement-fixing efficiency and precipitating ability of the 7S antibodies after 2-ME treatment was directly due to the reduction of the disulfide bonds or due to possible functional structural alterations in the complement-fixing and precipitating site(s) of gamma globulin resulting secondarily from the reduction of disulfide bonds.

These investigations of the functional roles of disulfide bonds shed some further light on the complement-fixing ability of pepsin-treated antibody, 5S RGG. This precipitating antibody may fix less complement (40 per cent) than 7S antibody (up to 100 per cent) because it has lost a number of the disulfide bonds in Porter fragment III which are important for complement-fixing ability and/or efficiency. In addition, we have shown that another disulfide bond, inter-chain disulfide bond [S—$S_{(1)}$], appears to be important for the complement-fixing efficiency of intact 7S RGG. The reduction of this bond in 5S RGG resulted in a loss of its aggregating ability, even when it had been previously aggregated with antigen. Reduction of this bond in 5S RGG (aggregated with antigen) also resulted in a total loss of its complement-fixing ability. Whether the loss of complement-fixing ability of 5S RGG was as a direct result of the reduction of this one inter-chain disulfide bond or secondary to the loss of its aggregating ability cannot be discerned from the present analysis.

SUMMARY

The number of total disulfide bonds in rabbit and sheep 7S gamma globulin, before and after treatment with 2-mercaptoethanol, has been measured by amperometric titration.

Mercaptan reduction could diminish the complement-fixing efficiency of 7S rabbit gamma globulin by no more than 90 per cent without any significant decrease in maximal complement-fixing ability. This was associated with the reduction of only 7 disulfide bonds, including 1 interchain disulfide bond [S—$S_{(1)}$].

The reduction of 7 disulfide bonds in 7S sheep gamma globulin was associated with a 64 per cent decrease in complement-fixing efficiency, while reduction of 10 to 11 disulfide bonds decreased the complement-fixing efficiency by 90 per cent. Reduction of more disulfide bonds was not associated with any further decrease in complement-fixing efficiency, nor of any decrease in maximal complement-fixing ability.

The reduction of more than 10 to 11 disulfide bonds in 7S rabbit and sheep gamma globulins was associated with a decrease in precipitating ability.

The disulfide bonds which are more labile to mercaptan reduction appear to be associated with complement-fixing efficiency while the disulfide bonds which are more resistant to mercaptan reduction appear to be associated with precipitating ability.

The measurement of interpolypeptide chain disulfide bonds has been discussed. One easily reduced interchain disulfide bond [S—$S_{(1)}$] appears to be important for the complement-fixing efficiency of 7S rabbit antibody. The integrity of this same bond is essential for the precipitating ability of 5S rabbit antibody and may also be important for its complement-fixing ability.

We gratefully acknowledge the kind assistance given us by Dr. E. L. Becker in these studies, and in the preparation of this manuscript.

BIBLIOGRAPHY

1. Edelman, G. M., and Benacerraf, B., On structural and functional relations between antibodies and proteins of the Gamma-system, *Proc. Nat. Acad. Sc.*, 1962, **48,** 1035.
2. Edelman, G. M., Unresolved problems in the analysis of the structure of antibodies, *in* Immunopathology, 3rd International Symposium, La Jolla, 1963, Basel, Benno Schwabe and Co., 1963, 57.
3. Grubb, R., and Swahn, B., Destruction of some agglutinins but not of others by 2 sulfhydryl compounds, *Acta Path. et Microbiol. Scand.*, 1958, **43,** 305.
4. Uhr, J. W., and Finkelstein, M. S., Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ϕX 174, *J. Exp. Med.*, 1963, **117,** 457.

5. Adinolfi, M., Polley, M. J., Hunter, D. A., and Mollison, P. L., Classification of blood-group antibodies as B_2M or gamma globulin, *Immunology*, 1962, **5,** 566.
6. Svehag, S.-E., and Mandel, B., The formation and properties of poliovirus-neutralizing antibodies. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction. II. 19S and 7S antibody formation: differences in antigen dose requirement of sustained synthesis, anamnesis, and sensitivity to x-irradiation, *J. Exp. Med.*, 1964, **119,** 1, 21.

6 *a*. Svehag, S.-E., The formation and properties of poliovirus-neutralizing antibody. III. Sequential changes in electrophoretic mobility of 19S and 7S antibodies synthesized by rabbits after a single virus injection, *J. Exp. Med.*, 1964, **119,** 225.

7. Bauer, D. C., Mathies, M. J., and Stavitsky, A. B., Sequences of synthesis of B_2-macroglobulin and γ_2-globulin antibodies during primary and secondary responses to proteins, *Salmonella* antigens, and phage, *J. Exp. Med.*, 1963, **117,** 889.
8. LoSpalluto, J., Miller, W. E., Jr., Dorward, B., and Fink, C. W., The formation of macroglobulin antibodies. I. Studies on adult humans, *J. Clin. Inv.*, 1962, **41,** 1415.

8 *a*. Fink, C. W., Miller, W. E., Jr., Dorward, B., and LoSpalluto, J., The formation of macroglobulin antibodies. II. Studies on neonatal, infants and older children, *J. Clin. Inv.*, 1962, **41,** 1425.

9. Fudenberg, H. H., and Kunkel, H. G., Physical properties of the red cell agglutinins in acquired hemolytic anemia, *J. Exp. Med.*, 1957, **106,** 689.
10. Fudenberg, H. H., Kunkel, H. G., and Franklin, E. C., High molecular weight antibodies, *in* Proceedings of the 7th Congress of the International Society of Blood Transfusion, Rome, 1958, (L. Hollander, editor) Basel, S. Karger, 1959, 522.
11. Ishizaka, K., Ishizaka, T., and Sugahara, T., Molecular bases of passive sensitization. I. Role of disulfide linkages in γ globulin molecule, *J. Immunol.*, 1961, **87,** 548.
12. Grey, H. M., Production of mercaptoethanol sensitive, slowly sedimenting antibody in the duck, *Proc. Soc. Exp. Biol. and Med.*, 1963, **113,** 963.
13. Uhr, J. W., Finkelstein, M. S., and Franklin, E. C., Antibody response to bacteriophage ϕ x 174 in non-mammalian vertebrates, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111,** 13.
14. Wiedermann, G., Miescher, P. A., and Franklin, E. C., Effect of mercaptoethanol on complement binding ability of human 7S gamma globulin, *Proc. Soc. Exp. Biol. and Med.*, 1963, **113,** 609.
15. Schur, P. H., and Becker, E. L., Pepsin digestion of rabbit and sheep antibodies. The effect on complement fixation, *J. Exp. Med.*, 1963, **118,** 891.
16. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961.
17. Porter, R. R., The structure of gamma globulin and antibodies, *in* Basic Problems in Neoplastic Disease, (A. Gellhorn, editor), New York, Columbia University Press, 1962.
18. Christian, G. D., and Schur, P. H., The amperometric titration of total and interchain disulfide bonds in gamma globulin, manuscript in preparation.

19. Cecil, R., and Wake, R. G., The reactions of inter and intra chain disulfide bonds in proteins with sulphite, *Biochem. J.*, 1962, **82,** 401.
20. Franek, F., and Lankas, V., The reactivity of disulphide bonds in pig γ-globulin, *Collection Czech. Chem. Commun.*, 1963, **28,** 245.
21. Palmer, J. L., and Nisonoff, A., Reduction and reoxidation of a critical disulfide bond in the rabbit antibody molecule, *J. Biol. Chem.*, 1963, **238,** 2393.
22. Relyveld, E. H., Toxiene et antitoxine Diptheriques, Etude Immunologique, Paris, Heremann et Cie, 1959.
23. Scheidegger, J. J., L'analyse immuno-electrophoretique des liquedes biologiques, *Bull. Soc. Chim. Biol.*, 1957, **39,** (suppl. 1), 45.
24. Nisonoff, A., Markus, G., and Wissler, F. C., Separation of univalent fragments of rabbit antibody by reduction of a single, labile disulfide bond, *Nature*, 1961, **189,** 293.
25. Karush, F., The role of disulfide bonds in the acquisition of immunologic specificity, *J. Ped.* 1962, **60,** 103.
26. Fleischman, J. B., Porter, R. R., and Press, E. M., The arrangement of the peptide chains in γ globulin, *Biochem. J.*, 1963, **88,** 220.
27. Porter, R. R., The hydrolysis of rabbit globulin and antibodies with crystalline papain, *Biochem. J.*, 1959, **73,** 119.
28. Smith, E. L., McFadden, M. L., Stockell, A., and Buettner-Janusch, V., The amino-acid composition of four rabbit antibodies, *J. Biol. Chem.*, 1955, **214,** 197.
29. Markus, G., Grossberg, A. L., and Pressman, D., The disulfide bonds of rabbit globulin and its fragments, *Arch. Biochem. and Biophysics*, 1962, **96,** 63.
30. Karush, F., Structural and energetic aspects of antibody-hapten interactions, *in* the Serological and Biochemical Comparisons of Proteins, (W. H. Cole, editor), New Brunswick, Rutgers University Press, 1958, 40.
31. Palmer, J. L., Nisonoff, A., and Van Holde, K. E., Dissociation of rabbit gamma globulin into subunits by reduction and acidification, *Proc. Nat. Acad. Sc.*, 1963, **50,** 314.
32. Osler, A. G., Quantitative studies of complement fixation, *Bact. Rev.*, 1958, **22,** 246.