The Disulphide Bonds of Human and Rabbit γ -Globulins

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1. The reaction of the disulphide bonds of the predominant species of human and rabbit γ -globulins (the 7s γ -globulins) with sulphite was studied in the presence and absence of denaturing agents and heavy-metal reagents. 2. The total number of bonds reacting/mol. of mol.wt. 160 000 was approx. 18 for human and 20 for rabbit γ -globulin. 3. Six S-S bonds/mol. of human and 6.5 S-S bonds/mol. of rabbit γ -globulin reacted with sulphite alone at pH 6. These appeared to include all the interchain S-S bonds. 4. The number of free SH groups was less than 0.2/mol. of human and less than 0.3/mol. of rabbit γ -globulin.

This paper describes the reactions of the disulphide (S-S) bonds of γ -globulins with sulphite:

This reaction is reversible and will only go to completion under conditions, e.g. at pH 7, when the concentration of RS^- is small (Stricks & Kolthoff, 1951; Cecil & McPhee, 1955). Under such conditions it is commonly used as a means of estimating S-S bonds, the extent of reaction being determined by estimation of the amount of thiol formed. It has also been observed that S-S bonds in native proteins show considerable differences in reactivity with sulphite (Kolthoff, Anastasi & Tan, 1958, 1959; Cecil & Loening, 1960; Cecil & Wake, 1962). Cecil & Wake (1962) showed that, whereas interchain bonds normally react with sulphite, intrachain bonds frequently require in addition the presence of a heavy-metal reagent or denaturing agent or both.

The object of the present work was, first, to use the sulphite reaction to estimate the total number of S-S bonds in γ -globulin, and, secondly, to see whether any differences in the reactivity of these bonds could be explained in terms of the ideas put forward by Cecil & Wake (1962). A necessary preliminary was to check on the number of free SH groups present before reaction with sulphite.

The preparations of γ -globulin used, whether from human or rabbit plasma, represent the predominant protein in the family of γ -globulins. This protein is often called 7s γ -globulin, γ_2 -globulin or immunoglobulin G. It is currently thought to consist of four polypeptide chains: two A (or H) chains, and two B (or L) chains (Porter, 1962). It is itself heterogeneous, although all its components

appear to contain some structure in common (e.g. Fahey, 1962; Kunkel, Allen, Grey, Martensson & Grubb, 1964). Results of estimations in terms of bonds/mol. therefore refer only to average values for the particular molecular populations.

MATERIALS

Human γ -globulin. This was prepared by chromatography on DEAE-cellulose (Peterson & Sober, 1958). Venous blood was drawn from laboratory personnel. The serum was passed through a column of Sephadex G-25 equilibrated with 30 mM-potassium phosphate buffer, pH 7.3 (KH_2PO_4 , 0.97 g./l.; K_2HPO_4 , 3.98 g./l.); the serum proteins are thus transferred into this buffer. The protein solution was then introduced into a column of DEAE-cellulose equilibrated with the same buffer at 25°, and was eluted with the buffer at this temperature. γ -Globulin (500–600 mg./100 ml. of serum) was delivered in a single narrow zone. The preparation was characterized as follows: (1) Ultracentrifugation in 0.1 M-KCl at pH 7.3: a single symmetrical boundary was obtained with $S_{20,w}$ 6.4s at a protein concentration of 12 mg./ml. (2) Immunoelectrophoresis (Grabar & Williams, 1953): a single band developed, corresponding to the cathodic two-thirds of the band of 7s γ -globulin given by normal human serum run concurrently.

Rabbit γ -globulin. This was a sample, given by Professor R. R. Porter, that had been prepared by chromatography on DEAE-cellulose in the cold room with 17.5 mM-sodium phosphate buffer, pH 6.5. He also gave us samples of the A and B peptide chains (prepared as described by Fleischman, Porter & Press, 1963), of the pieces I and III obtained by limited digestion with papain (Porter, 1959), and of that part of the A chain (A piece) contained in piece I. The relationships of these fragments to the intact γ -globulin molecule are discussed by Fleischman *et al.* (1963). The method used to prepare the A and B chains, and the A piece, involves reduction of interchain S-S bonds and alkylation of the SH groups thus formed; these alkylated groups are not detected by the titrations used in the present work. The specific extinction coefficients were supplied by Professor Porter: $E_{1\text{cm}}^{1\%}$ at 280 m μ for whole γ -globulin, 13.7;

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for A chain, 13.7; for B chain, 11.8; for piece I, 14; for piece III, 13; for A piece, 14.4. The A chain solutions were diluted with 0.5M-propionic acid, to remove opalescence, before their extinctions were read.

Chemicals. Phenylmercuric acetate (microanalytical reagent; British Drug Houses Ltd., Poole, Dorset) was dissolved in water to give 10mM final concentration. Dis-solution was slow, but complete after 16hr. at 60°. The molarity was checked by potentiometric titration against KBr at pH2 (Allison & Cecil, 1958), and found to agree with the weighed concentration.

Guanidine hydrochloride solutions (approx. 7M) were prepared by adding conc. HCl slowly to guanidine carbonate (analytical reagent; British Drug Houses Ltd.) at 0° to approx. pH3 and boiled to eliminate CO₂. These solutions gave no polarographic wave at the sensitivities used.

Sodium dodecyl sulphate (B.P. grade; L. Light and Co. Ltd., Colnbrook, Bucks.) was used as supplied.

All other reagents were of analytical grade.

METHODS

Estimation of free SH groups. The method of Allison & Cecil (1958) was used to estimate the number of SH groups present in the active protein and after denaturation with sodium dodecyl sulphate. Phenylmercuric acetate was used for the titrations instead of the hydroxide as it is now available commercially in a purified form.

Estimation of S-S groups. The method used was essentially that of Cecil & Wake (1962). The reactions with sulphite alone were carried out at 25° with 0.5M-Na₂SO₃ adjusted with dil. HNO₃ to pH7; they were stopped by adding 1ml. samples to 4ml. of 8M-urea in 0.3N-HNO₃ to give a final pH of 2.5 (the function of the urea was to prevent precipitation of the γ -globulin). The SH groups formed were estimated by amperometric titration with phenylmercuric acetate at -0.3v.

The total disulphide content was measured by carrying out the reaction in the presence of urea or guanidine hydrochloride; phenylmercuric acetate was also present in slight excess to prevent oxidation of the SH groups formed.

RESULTS

For both human and rabbit γ -globulins the number of S-S bonds/mol. have been calculated on the basis of a molecular weight of 160000, the value used by most previous authors. Recent work, however, suggests that this is too high (Pain, 1963). The molecular weights assumed for fragments of rabbit γ -globulin (Table 3) are those proposed by Professor Porter. In order that the more important results can be referred to revised molecular weights or extinction coefficients or both, they are also reported as μ moles of S-S bonds/100 'extinction units' of protein, where 1 extinction unit is that amount of protein which, in 1 ml. of solution, gives $E_{1\text{ cm.}}^{1\%}$ at 280m μ . Then μ moles of S-S bonds/100 extinction units $\times E_{1\text{ cm.}}^{1\%}$ at 280m $\mu \times \text{mol.wt.}/10^6$ = S-S bonds/mol.

Free SH groups

The number of SH groups in the active human γ -globulin was found to be less than 0.2/mol. The same result was obtained in the presence of 1% sodium dodecyl sulphate and 5M-guanidine hydrochloride. It was therefore concluded that few if any of the components of human γ -globulin contain free SH groups.

The same result was obtained with rabbit γ -globulin except that the number was less than 0.3/mol. Titrations of the SH groups in piece I and piece III showed less than 0.2group/fragment in both cases; the former was titrated in the presence and absence of 1% sodium dodecyl sulphate, the latter only in its presence. The A and B chains gave a zero titre, which was consistent with the fact that their preparation involved reaction with iodoacetamide.

Reaction of the S-S bonds with sulphite

Human γ -globulin. Human γ -globulin (0.05mM, i.e. 8mg./ml.) was allowed to react with 0.5M-sodium sulphite at pH6 and 25°. The time-course of the reaction is shown in Table 1, which gives the mean of two experiments. Maximum reaction occurs in approx. 3min. to give 6 S-S groups/mol. The slow decrease in the number of groups observed after longer reaction periods is likely to be due to oxidation of the SH groups formed.

To decide whether or not the interchain S-S bonds of the protein (Fleischman *et al.* 1963) had been broken by reaction with sulphite, the acidified reaction mixture was put through a column of Sephadex G-100 equilibrated with 0.5M-propionic acid. Peaks corresponding to the A and B chains were obtained (Fleischman, Pain & Porter, 1962). As judged by the extinction at 280m μ , the yield of protein in the B chain peak was 24%, close to the

Table 1. *Reaction of the S-S bonds of native human γ -globulin with sulphite*

The reaction was stopped by acidification at the times shown and the SH groups formed were estimated by titration with phenylmercuric acetate.

Reaction time	Number of S-S groups	
	(μ moles/100 extinction units of protein)	(groups/mol.)
1 min.	2.1 ₁	4.7
3 min.	2.7 ₀	6.0
10 min.	2.7 ₃	6.1
60 min.	2.4 ₆	5.5
24 hr.	1.0 ₁	2.2

theoretical maximum. This indicates that the bonds reacting with sulphite after 3 min. included all those linking the A and B chains.

Rabbit γ -globulin. Rabbit γ -globulin was allowed to react with sodium sulphite under the same conditions as above, but with only one reaction time, 10 min. The number of S-S groups reacting was $3.01 \mu\text{moles}/100$ extinction units of protein, or 6.6 groups/mol. Chromatography of the reaction mixture on Sephadex G-100 equilibrated with 1.0M -propionic acid indicated that all of the S-S bonds linking the A and B chains had been broken.

Reaction of the S-S bonds with sulphite in the presence of a denaturing agent

Human γ -globulin. Human γ -globulin (0.015M , i.e. 2.4mg./ml.) was allowed to react with 0.15M -sodium sulphite at pH 7 and room temperature for 15 min., in the presence of one of the following: 8M -urea, 5M -guanidine or 6M -guanidine. Ox insulin, which contains 3 S-S bonds/mol., was used as a control under the same conditions. The results obtained are given in Table 2. It would appear from these that human γ -globulin contains a total of approx. 18 S-S bonds/mol. Only 16 of these react in the presence of 8M -urea.

Rabbit γ -globulin and its fragments. These experiments were repeated with rabbit γ -globulin and its fragments, with 5M -guanidine as the denaturing agent. Once again reproducible results were obtained when the reactions took place in the presence of a small excess of phenylmercuric acetate. The results are given in Table 3.

DISCUSSION

The present estimates of SH groups and S-S bonds in whole γ -globulin are consistent with those obtained by previous workers using a variety of

methods (Markus & Karush, 1957; Putnam & Tan, 1960; Edelman & Poulik, 1961; Glazer & Smith, 1961; Markus, Grossberg & Pressman, 1962; Crumpton & Wilkinson, 1963; Franěk & Lankas, 1963). The finding that few, if any, of the components of rabbit γ -globulin contain SH groups is at variance with Markus *et al.* (1962), who observed an uptake of $2\text{mol. of Ag}^+/\text{mol. of protein}$, and took this to indicate the presence of 2 SH groups/mol.; but they could not confirm this with other agents reacting with SH groups. It is known too that the use of Ag^+ for SH estimations frequently gives rise to high results (Cecil, 1962).

The finding that urea is less effective than guanidine in rendering S-S bonds available for reaction with sulphite has been reported previously, for pig γ -globulin, by Franěk & Lankas (1963). That urea is less effective than guanidine (when both are near maximum concentration) in unfolding γ -globulin is suggested also by measurements of optical rotation: Buckley, Whitney & Tanford (1963) found it to be less effective as judged both by the maximum achievable increase in laevo-rotation and by the time required to reach this increase.

Experience with other proteins (Cecil & Loening, 1960; Cecil & Wake, 1962) and comparisons with amino acid analyses (Putnam & Tan, 1960; Crumpton & Wilkinson, 1963) suggest that all of the S-S bonds of γ -globulin react in the presence of 5M -guanidine and phenylmercuric ions.

After the reaction of human or rabbit γ -globulin with sulphite in the absence of a denaturing agent, chromatography in acid on Sephadex G-100 revealed that at least those interchain S-S bonds linking the A and B chains must have been broken. The possibility of some interchain S-S bonds remaining in A chain or B chain dimers is not excluded by this evidence, but is unlikely in view

Table 2. *Reaction of the S-S bonds of human γ -globulin with sulphite in the presence of a denaturing agent and phenylmercuric acetate*

The reactions were carried out at pH 7 and 25° for 15 min. Ox insulin was used as a control.

Denaturing agent	Number of S-S groups		
	Human γ -globulin		Ox insulin (groups/mol.)
	($\mu\text{moles}/100$ extinction units of protein)	(groups/mol.)	
8M -Urea	6.9 ₅	15.5	2.9 ₀
5M -Guanidine	8.0 ₂	17.8	2.9 ₅
6M -Guanidine	7.9 ₀	17.4	—

Table 3. *Reaction of the S-S bonds of rabbit γ -globulin and its fragments with sulphite in the presence of 5M -guanidine and phenylmercuric acetate*

The reaction was carried out at pH 7 and 25° for 15 min.

Protein	Assumed mol. wt.	Number of S-S groups	
		($\mu\text{moles}/100$ extinction units of protein)	(groups/mol.)
Whole γ -globulin	160000 (140000)	8.9 ₉	20 (17)
A chain	50000	5.1 ₀	3.5
B chain	20000	11.0	2.6
Piece I	42000	10.4	6.1
Piece III	50000	4.5 ₃	2.9
A piece	22000	5.7 ₆	1.8

of the known high reactivity of the S·S bond(s) linking the A and B halves of a γ -globulin molecule (Palmer & Nisonoff, 1964). Thus the S·S bonds cleaved can be taken to include all the interchain bonds present, and an upper limit to the number of interchain bonds is provided. The numbers of S·S bonds reacting with mercaptoethanol in the absence of a denaturing agent appear, from the results of Crumpton & Wilkinson (1963), to be about 1 bond/mol. less than have been found here to react with sulphite.

During the preparation of the A and B chains and A piece of rabbit γ -globulin at least the interchain S·S bonds were reduced and alkylated. The titrations of S·S bonds remaining in the chains and A piece therefore give a lower limit for the number of intrachain bonds present.

In the digestion of rabbit γ -globulin with papain, the piece I first appears as a dimer, which is halved when one of its S·S bonds is reduced by the cysteine present in the reaction mixture (Nisonoff, Markus & Wissler, 1961). The finding of no free SH group in piece I is therefore surprising; Markus *et al.* (1962) obtained the same result. Perhaps the protein remains linked by an S·S bond to the cysteine.

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