The Disulphide Bonds of the Heavy Chain of Rabbit Immunoglobulin G

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Six peptides containing eight half-cystine residues were isolated in good yield, after either oxidation or reduction and carboxymethylation of fragment C-1, which contains the *N*-terminal half of the heavy chain of rabbit immunoglobulin G. The sequences of five of these peptides had been reported previously (Cebra, Steiner & Porter, 1968b; Wilkinson, 1969) and that of the sixth was established. Other peptides containing half-cystine residues were isolated in much lower yield and are presumed to be derived from minor sequence variants. The cystine-containing peptides from enzymic digests of whole immunoglobulin G and Fc fraction were studied by several techniques and the results obtained enable us to put forward a scheme of the arrangement of the inter- and intra-chain disulphide bonds.

A sequence of 170 residues in the *N*-terminal 250 residues of the heavy chain of rabbit IgG‡ has been proposed (Cebra, Steiner & Porter, 1968b; Fruchter, Jackson, Mole & Porter, 1969; Wilkinson, 1969). In the present work the sequences around the half-cystine residues were investigated. This led to confirmation of some of the sequences given in the previous work, establishment of that of an additional 15 residues and has provided evidence for the positions of the intra- and inter-chain disulphide bonds of the molecule.

The numbering system used is based on that of the human myeloma IgG heavy chain Daw (Press & Hogg, 1969). The chains are aligned for maximum homology (Fruchter *et al.* 1969).

MATERIALS AND METHODS

Fragment C-1, the *N*-terminal half of the heavy chain of rabbit IgG, was prepared as described by Givol & Porter (1965). The tryptic peptides T1, T2 and T3 were prepared from reduced, carboxymethylated and trifluoroacetylated fragment C-1 as described by Cebra, Givol & Porter (1968a).

Preparation of completely reduced and [¹⁴C]carboxymethylated fragment C-1. Fragment C-1 (50 mg) was dissolved in 7M-guanidine hydrochloride–0.5M-tris-HCl

buffer, pH 8.4 (5 ml). Dithiothreitol (225 µl of a stock solution of 10 mg/ml) was added and the solution kept for 2 h at room temperature (Frangione & Milstein, 1968; Cebra *et al.* 1968a). Then iodo[2-¹⁴C]acetic acid (25 µl of a solution in water containing 64.5 µmol and 1 mCi/ml) was added and mixed well. After 2 min. unlabelled iodoacetic acid was added (0.45 ml of 0.1 M) and after a further 15 min the mixture was dialysed. This prior addition of the labelled iodoacetic acid results in preferential labelling of the protein thiol groups (J. M. Wilkinson, personal communication).

Labelling of partially reduced IgG. This was carried out as described by Frangione & Milstein (1968).

Cystine diagonal 'maps'. These were carried out at pH 3.5 by the method of Brown & Hartley (1963) on peptic-tryptic digests of whole IgG, fraction Fc and fraction C-1. Fraction C-1 was also similarly studied at pH 6.5 and 3.5 by using a tryptic-chymotryptic digest.

Identification of cystinylcysteinyl sequence in peptides. Samples (approx. 0.02–0.05 µmol) of dried peptides were oxidized with 200 µl of performic acid overnight at 0°C. After removal of the performic acid by drying, 400 µl of 10.5M-HCl was added and the mixture kept at 37°C for 3 days. In this way the peptide is converted into di- and tri-peptides (cf. Consden & Gordon, 1950). Cysteicysteinyl acid could then readily be recognized by paper electrophoresis at pH 1.9 (Sanger, Ryle, Smith & Kitai, 1955).

Digestion of rabbit IgG with pepsin. Rabbit IgG (200 mg) was dialysed for 16 h against 0.02M-HCl. The pH was adjusted to 2.0 and 8 mg of pepsin was added. It was digested for 16 h at 37°C, an additional 4 mg of pepsin being added after 4 h. The pH was maintained at 2.0. The mixture was freeze-dried and dissolved in 100% formic acid (5 ml) and loaded on Sephadex G-50 in 50% formic acid.

Other materials and methods. Enzymic digestions, separation of peptides by high-voltage paper electrophoresis and other methods and materials were as described by Press, Piggot & Porter (1966). Partial acid

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‡ Abbreviations: IgG, immunoglobulin G; CMCys, Cys and MetSO₂ (in amino acid sequences), carboxymethylcysteine, cysteic acid and methionine sulfoxide respectively.

hydrolysis of peptides with 0.03 M-HCl was carried out for 3 or 6 h at 105°C. Peptides were isolated by gradient elution from DEAE-Sephadex A-25 (10 ml fractions) by 300 ml of NH_4HCO_3 buffer, pH 8.5, at the concentrations described in the text, in each open chamber or by paper electrophoresis. The characterization of radioactive peptides was achieved by radioautography after purification by paper electrophoresis at pH 6.5, 3.5 and 1.9 or chromatography with butan-1-ol-acetic acid-water (Frangione & Milstein, 1968).

Radioautography. Radioautography with Kodak BB-54 X-ray film was carried out for 16–24 h after paper electrophoresis, each radioactive spot having 6000–20 000 c.p.m.

RESULTS

Cystine-containing peptides of fraction C-1: those not reduced by mild reduction. The peptides present in a tryptic-chymotryptic digest of completely reduced and carboxymethylated fraction C-1 ('intra'-chain disulphide bonds only were labelled) were separated by paper electrophoresis at pH 3.5. Four major areas, A, B, C and D, were recognized. The peptides in these areas were further purified by paper electrophoresis at pH 6.5 and 1.9. The mobilities of the major ones are given in Table 1, as are also their assignments to positions in the sequence given by Fruchter *et al.* (1969) and Wilkinson (1969). Peptides A1 and A2 were shown to be the predominant radioactively labelled peptides in a tryptic-chymotryptic digest of reduced and [^{14}C]-carboxymethylated peptide T3 prepared from peptides T2 plus T3 kindly supplied by S. A. Jackson (Cebra *et al.* 1968a). Peptide A3 was found to have the sequence CMCys-Ala-Arg. Peptide B was the only radioactively labelled peptide present in a tryptic-chymotryptic digest of reduced and [^{14}C]-carboxymethylated peptide T2. Peptide D2,

containing the CMCys-CMCys sequence, was not a major radioactively labelled peptide in a tryptic-chymotryptic digest of reduced and [^{14}C]-carboxymethylated peptide T3 prepared as described by Cebra *et al.* (1968b). However, the preparation used here was obtained under slightly different conditions from those described by them and it has been shown that the -Cys-Cys- residues are reduced, presumably not reproducibly, under both mild reduction ('inter'-chain bonds) and subsequent complete reduction ('intra'-chain bonds).

Cystine residues labelled by mild reduction and [^{14}C]-carboxymethylation of whole rabbit IgG. Below are listed the major radioactive peptides isolated by paper electrophoresis from a peptic digest of mildly reduced (only 'inter'-chain disulphide bonds broken) and [^{14}C]-carboxymethylated IgG.

Peptide 1 [m at pH 6.5 = -0.1, where m is mobility relative to aspartic acid (-1) with ϵ -DNP-lysine = 0]. This contains two carboxymethylcysteine residues and this, together with its composition, suggests it comes from the hinge region Lys-Val-Asp-Lys-Thr-Val-Ala-Pro-Ser-Thr-CMCys-Ser-Lys-Pro-Thr-Met-CMCys-Pro-Pro-Pro-Glu-Leu (carboxy-

methylcysteine residues at positions 221 and 227).

Peptide 2 (m at 6.5 = -0.68). Its composition suggests that this peptide has the sequence Pro-Leu-Ala-Pro-CMCys-CMCys-Gly-Asp-Thr-Pro-Ser-Ser-Thr and arises from peptide T3 (carboxymethylcysteine residues at positions 133 and 134). The mobility is in accord with the presence of three negative charges on the peptide. These carboxymethylcysteine residues are also labelled under conditions for 'intra'-chain reduction (see previous section).

Table 1. Major radioactive peptides isolated from a tryptic-chymotryptic digest of reduced and [^{14}C]-carboxymethylated peptide C-1 ('inter'-chain disulphide bonds not labelled) by paper electrophoresis at pH 3.5

Mobilities are expressed relative to aspartic acid (-1), the origin being taken as the position of ϵ -DNP-lysine. The mobility of peptide B suggests that Glx is Gln.

Peptide	Position of half-cystine residue	Mobility		Sequence implied from amino acid composition*
		At pH 3.5	At pH 6.5	
A1	146	0.73	0	Gly-CMCys-Leu-Val-Lys
A2	146	0.65	0	Related to peptide A1
A3	97	0.56	0	(CMCys,Ala,Arg)
B	201	-0.08	-0.31	Ser-Glx-Pro(Pro,Ser)Thr-CMCys-Asn-Val*
C1	22	-0.15	-0.29	Thr-Leu-Thr-CMCys-Thr-Val-Ala-Ser-Gly-Phe
D1a	22	-0.27	-0.34	Thr-CMCys-Thr-Val-Ala-Ser-Gly-Phe
D2	133-134	-0.27	-0.60	Ala-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-CMCys-CMCys-Gly-Asp-Thr-Pro-Ser-Ser-Thr-Val-Thr-Leu

* See Fruchter *et al.* (1969) and Wilkinson (1969).

Fig. 1. Fractionation of a tryptic digest of fragment C-1 that had been fully reduced and blocked with iodo-[^{14}C]acetate. A 100mg portion of the digest was put on a column (220cm $\times$ 3.0cm) of Sephadex G-50 in 0.05M- NH_3 . ---, E_{220} ; —, E_{280} ; ····, E_{230} ; - · - · -, radioactivity (c.p.m./ml of eluate). The fraction volume was 15ml. A 0.5ml sample of every alternate tube was taken for tryptic-chymotryptic digestion followed by electrophoresis at pH 3.5 and radioautography. Material in tubes 54–62 was taken for purification on DEAE-Sephadex A-25.

Peptide 3 (m at pH 6.5 = -0.38). This comes from the C-terminal end of the light chain and has the sequence Asn-Arg-Gly-Asp-CMCys (Frangione, 1969).

Isolation of tryptic peptide (Ts4) containing carboxymethylcysteinylalanylarginine from fragment C-1. It follows from the two previous sections that there is only one major previously unrecognized carboxymethylcysteine-containing peptide in reduced and carboxymethylated fragment C-1. It is one of those more difficult to reduce ('intra'-chain) and has the sequence CMCys-Ala-Arg. The isolation and determination of the sequence of the corresponding tryptic peptide, Ts4, is described below.

After fractionation of the tryptic digest of reduced and [^{14}C]carboxymethylated fraction C-1 (Fig. 1), one-thirtieth of each alternate tube was digested with trypsin plus chymotrypsin and radioautographs were taken after paper electrophoresis at pH 3.5 in order to identify the area where the tryptic peptide containing -CMCys-Ala-Arg- was. It was spread across the profile, which may suggest heterogeneity in this area of fraction C-1 (for instance some molecules may have a proline residue after the arginine, making the Arg-Pro bond not susceptible to trypsin). The tryptic peptide containing -CMCys-Ala-Arg- was isolated from fractions 54–62 (where it was most concentrated) by chromatography on DEAE-Sephadex A-25 with gradients of 0.005–0.2M-ammonium hydrogen carbonate

followed by a second gradient of 0.2–0.4M-ammonium hydrogen carbonate (all buffers were at pH 8.5). Peptide Ts4, which came off in the second gradient, was subsequently purified by rechromatography with a shallower gradient of 0.18–0.28M-ammonium hydrogen carbonate, pH 8.5. It was then purified by paper electrophoresis at pH 3.5 and pH 6.5. The peptide was also isolated in a similar manner from material from the peptide T4-T5 area shown in Fig. 2 of Cebra *et al.* (1968a), kindly supplied by Mr L. E. Mole.

The composition and sequence of peptide Ts4 is shown in Table 2. By homology with Daw protein (Press & Hogg, 1969) the CMCys residue of peptide Ts4 is placed in position 97.

Cystine bridges of the heavy chain of rabbit IgG recognized by using the diagonal method. Recognition of bridged cystine peptides by using the diagonal method of Brown & Hartley (1963) was attempted on tryptic-peptic digests of C-1 fraction, Fc fraction and whole IgG. Isolation and comparison of the amino acid composition of these peptides with known sequences in the N-terminal (cf. Fruchter *et al.* 1969; Wilkinson, 1969) and C-terminal (Hill, Lebovitz, Fellows & Delaney, 1967) halves of the heavy chain showed the existence of the following intra-chain bridges: residues 22–97 and 146–201 in the N-terminal half of the heavy chain and residues 262–322 and 368–426 in the C-terminal half of the chain. The bridges belonging to the Fc fragment were also

Table 2. *Peptides isolated from peptide Ts4*

Mobilities are expressed relative to aspartic acid (−1), the origin being taken as the position of ϵ -DNP-lysine. Peptide Ts4-Cd was present in small yield owing to incomplete chymotryptic digestion; presumably it is a mixture of (Phe,CMCys,Ala,Arg) and (CMCys,Ala,Arg).			
Peptide	Mobility at pH 6.5	Amino acid composition (residues/mol of peptide)	Dansyl-Edman results
Ts4	−0.23	Arg (0.9), CMCys (0.8), Asp (1.0), Thr (4.1), Ser (0.9), Glu (1.0), Pro (0.8), Ala (1.8), Ile (0.8), Tyr (0.9), Phe (0.9)	$\overrightarrow{\text{Ile}}(\text{Arg,CMCys,Asp,Thr}_4,\text{Ser,Glu,Pro,Ala}_2,\text{Tyr,Phe})$
Chymotryptic peptides from peptide Ts4			
Ts4-Ca	0	CMCys (1.0), Ala (1.0), Arg (1.0)	$\overrightarrow{\text{CMCys-Ala-Arg}}$
Ts4-Cb	−0.4	Asp (1.0), Thr (3.6), Ser (1.2), Glu (1.1), Pro (+), Ala (1.1), Ile (0.8), Tyr (0.8)	$\overrightarrow{\text{Ile-Thr-Ser-Pro-Thr}}(\text{Glu,Asp,Tyr,Thr}_2,\text{Ala})$
Ts4-Cc	0	Phe	Phe
Ts4-Cd	0	Phe (0.3), CMCys (+), Ala (1.0), Arg (1.0)	$\overrightarrow{\text{Phe}}(\text{CMCys,Ala,Arg})$
Peptides from a partial acid hydrolysate of peptide Ts4-Cb			
Ts4-Cb-PA1	−1	Asp	Asp
Ts4-Cb-PA2	0	Ile (0.7), Thr (2.0), Ser (1.0), Glu (1.0), Pro (0.8)	
Ts4-Cb-PA3	0	Ile (0.7), Thr (1.6), Ser (1.0), Pro (1.2)	
Peptides from a partial acid hydrolysate of peptide Ts4			
Ts4-PA1		Thr (2.0), Ala (2.0), Tyr (1.1), Phe (0.9), CMCys (0.6), Arg (1.0)	$\overrightarrow{\text{Thr-Ala-Thr-Tyr}}(\text{Phe,CMCys,Ala,Arg})$
Sequence of peptide Ts4			
Ile-Thr-Ser-Pro-Thr-Gln-Asp-Thr-Ala-Thr-Tyr-Phe-CMCys-Ala-Arg			

reported by Prahl & Porter (1968) and those in the Fd fragment are homologous with those reported in the human γ -chain (Frangione & Milstein, 1967). The pairings of the other half-cystine residues at positions 133-134 and 221 and 227 in the hinge area, and also the junction point to the light-chain C-terminal half-cystine, could not be determined by using the diagonal method.

Inter-chain cystine bridges of rabbit IgG containing the cysteinylcysteiny sequence. It was thought that, in a peptic digest of whole IgG, the peptide containing the -Cys-Cys- sequence might be joined to two other peptides and hence be separable by size from other cystine peptides, except perhaps those containing the inter-chain bonds. Hence we attempted to isolate this -Cys-Cys-containing peptide on a Sephadex G-50 column (220 cm $\times$ 3 cm diam.) (cf. Steiner & Porter, 1967). In the present work 50% formic acid was used as solvent to minimize peptide aggregation or adsorption. The profile (Fig. 2) was scanned for the whereabouts of peptides containing the -Cys-Cys- sequence by oxidation of samples from alternate tubes with performic acid and subsequent identification of cysteicystic acid in partial acid hydrolysates prepared with concentrated hydrochloric acid at 37°C. It was found that only tubes 30–34 yielded cysteicystic acid, with the maximum in tube 32. Some of the material in these tubes was completely reduced in guanidine hydrochloride and was [^{14}C]carboxymethylated.

Fig. 2. Fractionation of a peptic digest of rabbit IgG. A 200 mg portion of the digest was loaded on a column (220 cm $\times$ 3.0 cm) of Sephadex G-50 and eluted with 50% formic acid. The fraction volume was 15 ml. The profile (1 ml of each alternate tube) was scanned for the whereabouts of peptides containing the -Cys-Cys- sequence of the heavy chain and the C-terminal Gly-Asp-Cys sequence of the light chain. Fractions 30–34 (P) contained a peptide including the Cys-Cys and the Gly-Asp-Cys sequences. I indicates the position of elution of insulin on the same column.

After desalting on Sephadex G-15 in 0.05 M-ammonia and digestion with trypsin the peptides present were purified by paper electrophoresis at pH 3.5. Radio-

Table 3. *Radioactive tryptic peptides obtained by paper electrophoresis at pH 3.5 of the reduced and [¹⁴C]carboxymethylated fraction containing the -Cys-Cys- sequence isolated from a peptic digest of whole rabbit IgG (Fig. 2)*

Mobilities are expressed relative to aspartic acid (−1), the origin being taken as the position of ϵ -DNP-lysine. The peptic peptide corresponding to a1 was four residues longer at the *N*-terminus; that corresponding to a3 was two residues longer at the *N*-terminus.

Peptide	Position of half-cystine residue	Mobility		Sequence implied from amino acid composition*
		At pH 6.5	At pH 3.5	
a1	221 and 227	−0.34	+0.08	Thr-Val-Ala-Pro-Ser-Thr-CMCys-Ser-Lys-Pro- ^{Met} Thr-CMCys-Pro-Pro-Pro-Glu-Leu
a2	133 and 134	−0.68	−0.45	Pro-Leu-Ala-Pro-CMCys-CMCys-Gly-Asp-Thr-Pro-Ser-Ser-Thr
a3	C-Terminus of light chain	−1.1	−0.74	Gly-Asp-CMCys

* See Fruchter *et al.* (1969) and Frangione (1969).

Fig. 3. Predominant cystine peptides in a tryptic digest of peptic fragment (tubes 30–34 from experiment of Fig. 2) of rabbit IgG. The diagonal 'map' was prepared by electrophoresis of the digest at pH 3.5. The paper was then oxidized with performic acid vapour and run in a second dimension at pH 3.5. The broken lines enclose the area of peptides whose mobilities are not affected by the performic acid treatment. The positions of markers in each dimension are shown.

autography then showed the presence of several spots, three of which stood out in intensity. All of the radioactive spots were purified by paper electrophoresis at pH 6.5, eluted and analysed. All of the minor bands were derived from the major peptides listed in Table 3. These major ones were those containing (1) the hinge-region peptide with carboxymethylcysteine residues at positions 221 and 227, (2) the -Cys-Cys-containing peptide from

peptide T3 with carboxymethylcysteine residues at positions 133 and 134 and (3) those involved in the C-terminal sequence of the light chain, Gly-Asp-CMCys.

Some of the material in tubes 30–34 (Fig. 2) was digested with trypsin and a diagonal 'map' prepared at pH 3.5. The predominant peptides of the diagonal 'map' were the three listed above as shown in Fig. 3. Analyses were as follows. Peptide 1: Cya (1.0), Asp (1.0), Gly (1.0); therefore Gly-Asp-Cya from the light chain. Peptide 2: Cya (2.0), Asp (1.0), Thr (2.1), Ser (1.9), Pro (3.3), Gly (1.0), Ala (1.0), Leu (1.2); therefore Pro-Leu-Ala-Pro-Cya-Cya-Gly-Asp-Thr-Pro-Ser-Ser-Thr for residues 129–141 in the heavy chain. Peptide 3: Cya (2.0), Thr (3.2), Ser (2.1), Glu (1.1), Pro (5.6), Ala (1.0), Val (0.9), Leu (1.0), MetSO₂ (0.15); therefore Thr-Val-Ala-Pro-

Ser-Thr-Cya-Ser-Lys-Pro-^{Thr}Met-Cya-Pro-Pro-Pro-

Glu-Leu for residues 215–235.

The presence of the peptic peptide containing the sequence -Gly-Asp-Cys was investigated by taking 1 ml portions from each alternate tube, oxidizing with performic acid and digesting with trypsin. Paper electrophoresis at pH 6.5 was used to show the presence of the peptide Gly-Asp-Cya, which has a unique mobility of 1.1 and stains yellow with ninhydrin-collidine. It was obtained only from tubes 30–34.

The elution volume of 25 mg of ox insulin in 50% formic acid on the Sephadex G-50 column was at tubes 48–49 (Fig. 2). It can thus be concluded that the piece of IgG in the peptic digest has a higher molecular weight than that of insulin (mol.wt. 5700).

Existence of minor variants in fragment C-1. Several pieces of information suggest that there are minor variants associated with the cysteine residues

of fragment C-1. These are treated in order as follows.

(a) Extra carboxymethylcysteine-containing peptide in fraction C-1. Two acidic radioactive peptides were isolated from regions near the break-through point in a tryptic-chymotryptic digest of reduced and [^{14}C]carboxymethylated fraction C-1 separated on a Sephadex G-50 column in 0.05 M-ammonia. In this system it appears that peptides of higher net negative charge move fastest down the Sephadex column. One of these was peptide D2 (see Table 1). The other was purified on DEAE-Sephadex A-25 and by paper electrophoresis at pH 6.5. It has the analysis CMCys (+), Asp (1.2), Thr (3.7), Ser (1.2), Glu (1.0), Pro (0.9), Gly (0.3), Ala (0.9), Val (0.1), Ile (0.6), Leu (0.2), Tyr (0.9), Phe (0.2), and it was recovered in significant yield. The *N*-terminal amino acid was found to be isoleucine and carboxypeptidase A showed that tyrosine was *C*-terminal and followed by threonine and alanine. This information suggests that the peptide arises from a peptide analogous to the Ts4 peptide and has the sequence

$\xrightarrow{\text{Ile}}$ (CMCys, Thr, Ser, Pro, Thr, Glu, Asp, Thr) Ala-Thr-
 $\xleftarrow{\text{Tyr}}$ expected from a chymotryptic split of peptide
 Ts4. The insertion of a carboxymethylcysteine
 residue is justified by the radioactivity of the pep-
 tide and its mobility at pH 6.5 ($m = -0.48$). Diges-
 tion with subtilisin gave a radioactive spot (m at
 pH 6.5 = -0.73) of composition (CMCys, Glu, Asp-
 Thr, Ala, Thr, Tyr). This information suggests that
 a percentage of the molecules in fraction C-1 has an
 extra carboxymethylcysteine residue in this area.

(b) Unidentified peptides in IgG. In a peptic digest of mildly reduced and [¹⁴C]carboxymethylated IgG small amounts of two unidentified peptides were obtained. They had the following amino acid composition: (CMCys, Asp₂, Ser, Glu, Gly, Val, Phe, Arg) (*m* at pH 6.5 = -0.21) and (CMCys, Asp_{0.9}, Thr, Ser₂, Glu_{0.7}, Pro, Gly, Ala, Val_{1.4}) (*m* at pH 6.5 = -0.21).

(c) Amino acid compositions and specific radioactivities of tryptic fragments T2 and T3 from fraction C-1. The two peaks found in peptide T2 (Fig. 4 of Cebra *et al.* 1968a) were found in the present work to have very different specific radioactivities, considered on the basis of either peptide or carboxymethylcysteine, and also differing contents of carboxymethylcysteine, the values being 2.6 and 1.9 mol/mol of peptide T2 (assumed to have 70 residues). Further, it was shown that peptide T3 could be spread by gradient elution from DEAE-Sephadex A-25 into three incompletely separated peaks of different specific radioactivity and carboxymethylcysteine contents; in fact the analytical results were 4.6, 4.0 and 3.8 mol of carboxymethylcysteine for the three successive peaks (peptide T3 was taken to contain 76 residues). This suggests

Fig. 4. Structure of peptide isolated from a peptic digest of rabbit IgG. It is shown as a monomer, though isolated as a dimer joined by the inter-heavy-chain bond from cystine residue 227. The numbering is that used for Daw-protein heavy chain, but the deletions assumed in the rabbit sequence to give maximum homology (Fruchter *et al.* 1969) have been omitted.

that there is an extra half-cystine residue in peptide T3 (or more probably several fractional half-cystine residues) that has not been identified in position in the sequence.

Continuation of peptide Ts4 at the C-terminal end. Attempts were made to obtain C-terminal overlap peptides of peptide Ts4 by preferential release of aspartic acid residues by hydrolysis with dilute hydrochloric acid (Schultz, 1967) from reduced and [^{14}C]carboxymethylated fraction C-1. Several fractions containing -CMCys-Ala-Arg as marker peptide were examined. In all cases it was not possible to obtain pure peptides as judged by amino acid analysis, although they all seemed to have (from amino acid composition only) the starting sequence Thr-Ala-Thr-Tyr-Phe-CMCys-Ala-Arg- (positions 92-99). These findings, taken in conjunction with those of Fruchter *et al.* (1969) from chymotryptic peptides in this area, agree with the postulate of a highly variable region commencing at position 100.

DISCUSSION

Analyses suggest that there are 7.4-8 half-cystine residues in fragment C-1 (Cebra *et al.* 1968a). The findings of eight major half-cystine residues by the radioactive-labelling and diagonal-electrophoresis experiments reported above suggests that these account mainly for the half-cystine residues of this section of heavy chain. However, there are other minor partial half-cystine residues present. Their presence in the peptide Ts4 region and probably also in peptide T3 is due to the known variability of sequence in the N-terminal 115 residues (Press & Hogg, 1969), but the minor sequences in peptide T2

known to be in the constant part of the chain would be expected to arise from allotype or subclass changes.

Among the tryptic peptides of trifluoroacetylated fragment C-1 (after complete reduction and carboxy-methylation) was one named T1, which Cebra *et al.* (1968a) suggested might be derived from the N-terminal end of the heavy chain. During the present work the cystine-containing peptides were examined and it was clear that most of those found in the whole of fragment C-1 were present in peptide T1. It appears therefore that the T1 fraction is formed in part from aggregated material derived from different sections of the peptide chain.

The diagonal technique gave unequivocally the positions of four intra-chain disulphide bonds in the heavy chain, confirming the two in the Fc fraction reported by Prahl & Porter (1968) and establishing the position of two in the Fd region as shown in Fig. 5. The peptide containing the Cys-Cys sequence could not, however, be identified, and there was uncertainty as to its relation to the other two half-cystine residues near the centre of the chain and to the light-chain peptide joined by the heavy-light inter-chain bond.

Peptic digestion of IgG gave a large-molecular-weight fraction that, after tryptic digestion, was shown to contain the three distinct sequences shown in Fig. 4. That they were covalently bonded to each other was likely as they remained together in a strongly dissociating solvent (50% formic acid), and confirmation of this was given by the diagonal technique. It was also clear that they were present as dimers, as they were eluted from a Sephadex column in 50% formic acid well ahead of insulin

Fig. 5. Proposed scheme of the arrangement of the inter-chain and intra-chain disulphide bonds of the heavy chain of rabbit IgG. The numbering is that used for the Daw-protein heavy chain.

(mol.wt. 5700 in that solvent). These peptides contain 39 residues and hence would have a molecular weight of about 4000 as a monomer and 8000 as a dimer.

Prahl, Mandy, David, Steward & Todd (1969) took advantage of the methionine residue present in the A11 allotype heavy chains at position 226 and showed by digestion with cyanogen bromide that there was only one disulphide bond between the heavy chains and that it was at position 227. If this is accepted, only one arrangement of the other disulphide bonds is possible that will hold each peptide covalently bonded to the other, and this is as shown in Fig. 4. This arrangement is in agreement with the finding in the present work that the Fab fragment does not contain cysteine residue 227, and hence the bond joining the two heavy-chain peptides must be between residue 133 or 134 and residue 221 unless disulphide-bond interchange occurred during the preparation of the Fab fragment. It has not been possible to distinguish between the residues 133 and 134.

The complete arrangement of the inter-chain disulphide bonds of rabbit IgG and the intra-chain disulphide bonds of the heavy chain are shown in Fig. 5. It is in agreement with the early evidence of Palmer & Nisonoff (1964) that there was only one inter-heavy-chain bond in rabbit IgG. It fits well with the results of Frangione, Milstein & Pink (1969), who showed that in human $\gamma 2$, $\gamma 3$ and $\gamma 4$ IgG the heavy chain-light chain bond was to residue 133 whereas in $\gamma 1$ IgG it was known to be to residue 221 (Steiner & Porter, 1967; Frangione & Milstein, 1967). Unless there was a major structural difference between the subclasses it was likely that the residues 133 and 221 would be sterically close, and the direct demonstration that in rabbit IgG residue 133 or 134 is joined to residue 221 proves this point. The failure to detect any other arrangement of disulphide bonds in the rabbit IgG suggest that, in the pooled material used, any subclasses present in significant content have the same inter-chain disulphide bond arrangement.

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