Hardwick, D.C. & Linzell, J.L. (1960). *J. Physiol.* **154**, 547.
Hardwick, D. C., Linzell, J. L. & Price, S. M. (1961). *Biochem. J.* **80** 37.
Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
Kisin, I. E. & Tsaturov, Y. L. (1961). *Bull. exp. Biol. Med., U.S.S.R. (Engl. trans.)* **50**, 864.
Kleiber, M. (1954). *Rev. Canad. Biol.* **13**, 333.
Kleiber, M., Black, A. L., Brown, M. A., Baxter, C. F., Luick, J. R. & Stadtman, F. H. (1955). *Biochim. biophys. Acta*, **17**, 252.
Lindsay, D. B. (1959). *Vet. Rev.* **5**, 103.
Lintzel, W. (1934). *Lait*, **14**, 1125.
Linzell, J. L. (1960). *J. Physiol.* **153**, 492.
Lucas, J. M., Kaneko, J. J., Hirohara, K. & Kleiber, M. (1959). *Agric. Fd Chem.* **7**, 638.
Luick, J. R. (1960). *J. Dairy Sci.* **43**, 1344.
Luick, J. R. & Kleiber, M. (1961). *Amer. J. Physiol.* **200**, 1327.
McArdle, B. A. (1955). *Biochem. J.* **60**, 647.
Macy, I. G., Kelly, H. J. & Sloan, R. E. (1953). *The Composition of Milks.* Washington: National Academy of Science National Research Council, Publication no. 254.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Peirce, E. C. (1960). *J. thorac. Surg.* **39**, 438.
Popják, G., Folley, S. J. & French, T. H. (1951). *Biochem. J.* **48**, 44.
Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.
Riis, P. M., Luick, J. R. & Kleiber, M. (1960). *Amer. J. Physiol.* **198**, 45.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Spencer, A. F. & Lowenstein, J. M. (1962). *J. biol. Chem.* **237**, 3640.
Srere, P. A. & Lipmann, F. (1953). *J. Amer. chem. Soc.* **75**, 4874.
Tombropoulos, E. G. & Kleiber, M. (1961). *Biochem. J.* **80**, 414.
Utter, M. F. (1959). *Ann. N.Y. Acad. Sci.* **72**, 451.
Utter, M. F. & Keech, D. B. (1960). *J. biol. Chem.* **235**, PC17.
Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.
Wood, H. G., Gillespie, R., Hansen, R. G., Wood, W. A. & Hardenbrooke, H. J. (1959). *Biochem. J.* **73**, 694.

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The Arrangement of the Peptide Chains in γ-Globulin

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Although *N*-terminal amino acid analysis of γ-globulin suggested that the number of peptide chains/molecule depended on the species of origin (Porter, 1960), other workers (Edelman, 1959; Edelman & Poulik, 1961; Franěk, 1961; Ramel, Stellwagen & Schachman, 1961) showed that, with all the γ-globulins examined, reduction in 6 M-urea led to a uniform fall in molecular weight from about 150 000 to about 50 000. This evidence indicated that all γ-globulins have the same number of chains/molecule and that therefore some of the *N*-terminal amino acids must be unreactive. The products obtained after reduction in urea are insoluble except in urea solutions, and hence are difficult to fractionate and have lost all biological activity. It was observed, however (Porter, 1962; Fleischman, Pain & Porter, 1962), that, when γ-globulin was reduced with mercaptoethanol in the absence of urea, five out of twenty disulphide bonds were broken, and that, though the molecular weight at neutral pH was unchanged, acidification caused the dissociation into two products which could be separated on Sephadex G-75 columns with 100% recovery and which were soluble and retained biological activity. The molecular weights of the two components (Pain, 1963) indicated that there were two larger chains A and two smaller chains B in a molecule of γ-globulin. As chains A and B retained their antigenic activity their relation to the three pieces formed by papain digestion of γ-globulin (Porter, 1959) could be established and led to the postulation of a diagrammatic structure for γ-globulin (Porter, 1962), shown in Scheme 1. The papain-digestion pieces I and II both contain an antibody-combining site and are considered to be identical in their general structure (Palmer, Mandy & Nisonoff, 1962; Stelos, Radzimski & Pressman, 1962).

We now describe a more detailed characterization of the products of reduction of whole γ-globulin and of the papain-digestion pieces. The results substantiate most of the features of the four-chain structure, the location of papain hydrolysis and the position of the antibody-combining site.

EXPERIMENTAL

Materials

γ-Globulin from horse, human and rabbit serum was prepared by chromatography on diethylaminoethylcellulose (Sober & Peterson, 1958). Some preparations of rabbit γ-globulin were also made by precipitation with Na_2SO_4

(Kekwick, 1940). No significant difference between the two types of preparation of rabbit γ-globulin was observed. All preparations appeared to be free of impurities when examined by starch-gel electrophoresis at pH 8·6 (Smithies, 1955). Rabbits were immunized as described by Press & Porter (1962) with human γ-globulin, bovine serum albumin, human serum albumin, ovalbumin and *Pneumococcus* type 3. A horse was immunized with rabbit γ-globulin by Miss M. Barr of the Wellcome Research Laboratories, Beckenham, Kent. The γ-globulin was prepared for injection by mixing equal volumes of a 1% (w/v) solution of globulin and 10% (w/v) potassium alum, and adjusting the pH to 6·8 with 2N-NaOH. To this were added 0·1 vol. of Arlacel (Honeywell and Stein Ltd.) and 0·9 vol. of Bayol F (Esso Petroleum Co. Ltd.), and the mixture was emulsified. The immunization schedule was as follows: on 5. vi. 61, 10 ml. of mixture given intramuscularly; on 26. vi. 61, 8 ml. of mixture given intramuscularly; on 12. vii. 61, 8 l. bleeding; on 17. vii. 61, 8 l. bleeding; on 12. ix. 61, 25 ml. of mixture given intramuscularly; on 26. x. 61, 50 ml. of mixture given intramuscularly; on 6. xi. 61, final bleeding. The 17. vii. 61 blood contained 5 mg. of antibody/ml., and the 6. xi. 61 blood 8 mg. of antibody/ml. Horse anti-(diphtheria toxoid) serum was a gift from the Wellcome Research Laboratories. It contained 900 i.u./ml., and with optimum concentration of toxoid gave 12 mg. of precipitate/ml. Diphtheria toxoid was a purified preparation given by Dr L. B. Holt.

Methods

Reduction and fractionation of γ-globulin was carried out as described by Fleischman *et al.* (1962), except that degassing and saturation of the solution with nitrogen before the addition of mercaptoethanol was discontinued as it appeared to have no advantage. The concentration of mercaptoethanol used for reduction was 0·2M, except when the immunological activity of the peptide chains from horse antibody was examined when 0·06M-mercaptoethanol was used.

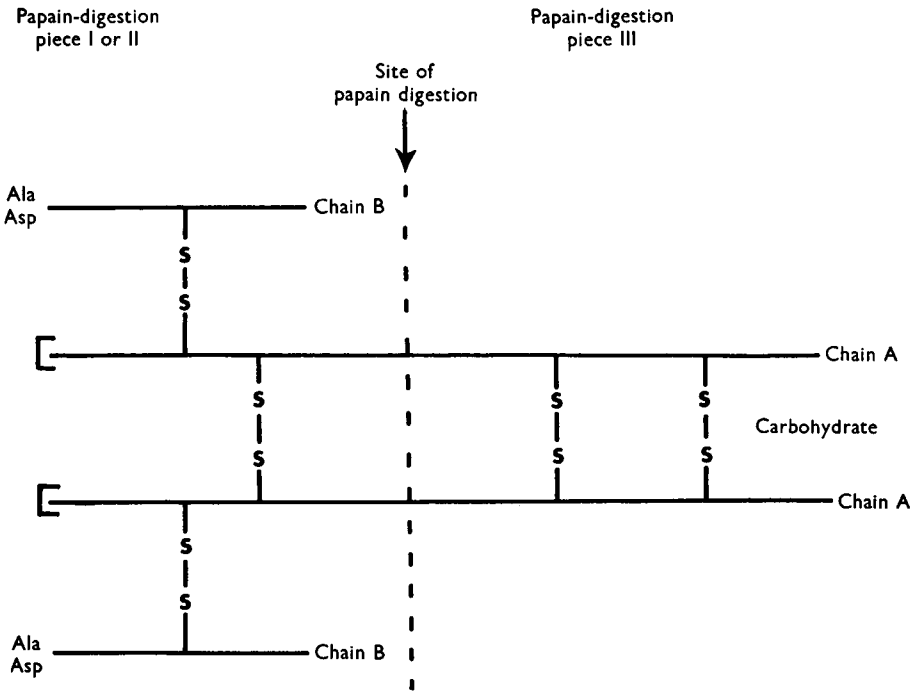
N-Terminal amino acids were determined by the method of Sanger (Porter, 1957).

Papain digestion of γ-globulin and isolation of the products was carried out as described by Porter (1959).

Neutral sugars were identified by paper chromatography. A sample containing 80–150 μg. of hexose was hydrolysed in 0·5N-HCl for 16 hr. at 100°, the hydrolysate diluted to 5 ml., and treated with Dowex 1 (OH^- form) to remove the HCl and then with Dowex 50 (H^+ form) to remove the peptides and amino acids. The resulting eluate, which contained only neutral sugars, was evaporated on a rotary still under reduced pressure and chromatographed on Whatman no. 1 paper for 20 hr. with butan-1-ol–pyridine–water (6:4:3, by vol.). The sugars were detected by spraying with the *p*-anisidine–phthalate reagent described by Rosevear & Smith (1961).

The quantitative determination of hexose was by the orcinol technique as described by Winzler (1955), with mannose as standard; the extinction was read at 505 mμ.

Hexosamine was determined by Cessi's modification of the Elson–Morgan method, as described by Johansen, Marshall & Neuberger (1960). The optimum hydrolysis time was 6 hr. at 100° in 4N-HCl, except for the glycopeptide, which was hydrolysed for only 3 hr. The hydrolysates were evaporated *in vacuo* in a desiccator at room temperature over P_2O_5 and KOH, and hexosamine was determined with glucosamine as standard.



Scheme 1. Diagrammatic structure of rabbit γ-globulin (Porter, 1962).

Sialic acid was determined by the thiobarbituric acid method, as described by Warren (1959), after hydrolysis in 0·1 N-H_2SO_4 for 2 hr. at 80°. *N*-Acetylneuraminic acid was used as standard.

Fucose was determined by the cysteine–H_2SO_4 method of Dische & Shettles (1948), with L-fucose as standard. A blank without cysteine was found to be satisfactory to correct for the colour contributed by the protein. To correct for the colour produced by galactose and mannose, the extinctions at 420 mμ were subtracted from those at 397 mμ.

Antibody activity was followed qualitatively by the delay in precipitation caused on the addition of active fragments to antiserum and antigen in optimum amounts. Polarization of fluorescence, with an antigen coupled to a fluorescent dye, was also used (Dandliker & Feigen, 1961). Quantitative determinations of antibody activity were based on co-precipitation or inhibition of precipitation as described by Kabat & Mayer (1961).

Electrophoresis in starch gel in 8 M-urea and formate buffer, pH 3·5, was carried out as described by Edelman & Poulik (1961).

RESULTS

Reduction of whole γ-globulin. Fleischman *et al.* (1962) have shown that the two components, A and B, obtained after reduction of γ-globulin and fractionation on Sephadex G-75 in N-propionic acid or N-acetic acid, were obtained with 95–100% recovery and in relative yields: A, 73–75%, and B, 25–27%. To interpret this result in terms of structure it was necessary to establish that the components could not be split further and that they were free of each other. Complete reduction with mercaptoethanol in 10 M-urea did not alter the molecular weights of A and B nor their behaviour on electrophoresis on starch gel in 8 M-urea. It was unlikely therefore that either consisted of smaller peptide chains still held together by disulphide bonds. When collagen is heated in M-hydrazine at pH 10 at 37° for 1 hr. it fragments, and evidence has been offered suggesting that ester-like bonds are split under these conditions (Blumenfeld & Gallop, 1962). Similar treatment of chains A and B caused no detectable decrease in size.

The contamination of each chain by the other was determined by different methods applicable to γ-globulins of different species. With human γ-globulin and horse γ-globulin the chains A and B separate cleanly on electrophoresis on starch gel in 8 M-urea with formate buffer, and hence impurities could be detected. With rabbit γ-globulin the chains A and B overlap on electrophoresis under these conditions, and hence contamination of one by the other could not be observed (Fleischman *et al.* 1962). However, in this species all the *N*-terminal alanine and aspartic acid of the original molecule are present in chain B (Fleischman *et al.* 1962), and as judged by this criterion rerun preparations of chain A contained not more than 3% of chain B.

Also Lawler (quoted by Cohen, 1963) has shown that the *Inv* and *Gm* characters of human γ-globulin are on chains B and A respectively, and in this respect no contamination of either chain by the other could be detected.

To establish further the purity of the chain A preparation, reduced rabbit γ-globulin was run on a Sephadex G-200 column in N-propionic acid (Fig 1). Four peaks appeared of which only the last contained chain B, suggesting considerable complexity for chain A. However, when the same experiment was repeated with 8 M-urea–N-propionic acid as solvent, the results resembled those obtained with Sephadex G-75, namely a major and a minor peak, except that at the solvent front there was another small peak of aggregated material. The minor peak is probably the aggregated chain A always seen in ultracentrifuge patterns and running more slowly than unreduced γ-globulin when subjected to electrophoresis on starch gel in 8 M-urea. The first three peaks on the Sephadex G-200–N-propionic acid column were indistinguishable when subjected to electrophoresis under these conditions and gave a typical chain A pattern. Hence the apparent complexity of chain A in N-propionic acid is probably due to association in this solvent and not to the presence of other products. In the fractionation of reduced rabbit γ-globulin on Sephadex G-200 in 8 M-urea–N-propionic acid, the yield of chain B was 26% of the total protein, in

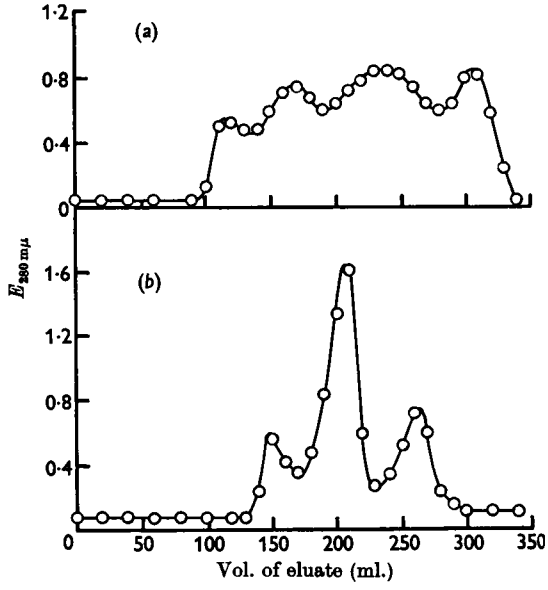


Fig. 1. Fractionation of reduced rabbit γ-globulin on a Sephadex G-200 column (65 cm. × 2·8 cm.) in (*a*) N-propionic acid and (*b*) 8 M-urea–N-propionic acid. Experimental details are given in the text.

agreement with the yields of chain B from Sephadex G-75 columns in N-propionic acid.

Amino acid analyses of whole rabbit γ-globulin and of rabbit-γ-globulin chains A and B have been given by Crumpton & Wilkinson (1963). The values have been recalculated as moles/mole in Table 1, and the molecular weights taken are those found for the horse-γ-globulin chains (Pain, 1963). Although a detailed study of the molecular weights of the rabbit-γ-globulin chains was not undertaken owing to the persistence of aggregates in preparations of chain A, chains B of horse γ-globulin and of rabbit γ-globulin have the same molecular weight and chains A have the same sedimentation coefficient. The greatest difference between the amino acid contents of chains A and B is in the histidine, arginine, proline, alanine, methionine, tyrosine and *S*-carboxymethylcysteine. These all differ by at least 40%. It appears that there is one SH group/chain B and four SH groups/chain A, giving a total of ten SH groups/molecule in reduced γ-globulin, in agreement with polarographic data.

The carbohydrate analyses are summarized in Table 2. Of the total carbohydrate 95% was present in chain A. The small amounts of carbohydrate present in chain B could have arisen from contamination with chain A, but they could be an integral part of some molecules of chain B, as this chain is obviously heterogeneous by charge and other criteria. Paper chromatography showed that the hexose consists of mannose and galactose in a ratio of about 2:1, together with a trace of fucose.

Reduction of papain-digestion pieces I and II. The proposed structure (Scheme 1) suggested that, if papain-digestion pieces I or II were reduced under conditions similar to those used for whole γ-globulin, then two fractions should be obtained, namely A piece from the *N*-terminal end of chain A and B piece, and these two fragments should be of approximately equal size. The elution diagrams of papain-digestion piece I and of reduced papain-digestion piece I on Sephadex G-75 in N-propionic acid are shown in Fig. 2. Some aggregation is apparent in both but in reduced papain-digestion piece I there were also two peaks of approximately equal area. The slower moved at the same position as chain B. This was isolated and was found to have the same *N*-terminal amino acids as chain B, to be antigenically indistinguishable from chain B

Table 1. *Amino acid analyses of rabbit γ-globulin and of the constituent peptide chains A and B*

The results were calculated from the data of Crumpton & Wilkinson (1963) by assuming the molecular weights for the horse-γ-globulin peptide chains A and B (Pain, 1963) to be 50 000 and 20 000 respectively, and that of whole γ-globulin to be 140 000.

Amino acid	Amino acid content (moles/mole)			
	Chain A	Chain B	2 Chains A + 2 chains B	γ-Globulin
Lys	23	7·8	62	63
His	6·5	1·3	16	18
Arg	16	2·6	37	39
Asp	33	16	98	106
Thr	49	24	146	144
Ser	49	19	136	135
Glu	40	18	116	120
Pro	36	10	92	99
Gly	33	16	98	98
Ala	24	13	74	73
Val	41	18	118	118
Met	4·6	0·5	10	12
Ileu	15	6·2	42	43
Leu	30	10	80	84
Tyr	17	9·6	53	54
Phe	14	5·2	38	39
CyS	9·3	5·5	29	36
$CyS{\cdot}CH_2{\cdot}CO_2H$	3·7	1·0	9·4	—
Try	7·8	1·9	20	22

Table 2. *Carbohydrate contents of rabbit γ-globulin and of the constituent peptide chains*

Experimental details are given in the text. The assumed molecular weights of A chain, B chain and whole γ-globulin were 50 000, 20 000 and 140 000 respectively (see Table 1).

Carbohydrate	Carbohydrate content (moles/mole)			
	Chain A	Chain B	2 Chains A + 2 chains B	γ-Globulin
Hexose	4·5	0·27	9·5	9·6
Hexosamine	4·0	0·16	8·3	8·2
Sialic acid	0·41	0 (< 0·01)	0·8	0·9

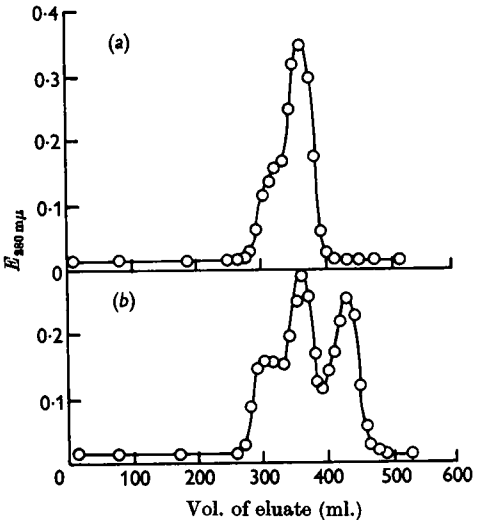


Fig. 2. Fractionation of reduced papain-digestion piece on a Sephadex G-75 column (120 cm. × 2·8 cm.) in N-propionic acid. (*a*) Unreduced piece I; (*b*) reduced piece I. Experimental details are given in the text.

(Fig. 3) and to resemble chain B very closely by amino acid analysis (Crumpton & Wilkinson, 1963). It was therefore identified as chain B. The other component should have been A piece but moved as if it had the same molecular weight as whole papain-digestion piece I. However, when the molecular weight was determined in 8 M-urea, pH 3·5, it was found to be about 22 000, whereas that of papain-digestion piece I is 40 000 in 8 M-urea as in aqueous solution (Pain, 1963). The A piece also differed from papain-digestion piece I in that it had a very low *N*-terminal amino acid content as had whole chain A. This suggests that A piece is dimerized in N-propionic acid and hence could be separated from B piece. The antigenic behaviour of A piece with goat anti-(papain-digestion piece I) is shown in Fig. 3. It was distinct from B piece and fused with A chain, showing that the antigenic sites with respect to this antiserum were common to chain A. There was also an additional faint line possibly arising from a degradation product. Amino acid analyses of A and B pieces and whole papain-digestion piece II are compared in Table 3. The A piece contains two *S*-carboxymethylcysteine residues/molecule and B piece one, in agreement with the diagrammatic structure (Scheme 1). There are also considerable differences in the content of arginine, glutamic acid and methionine.

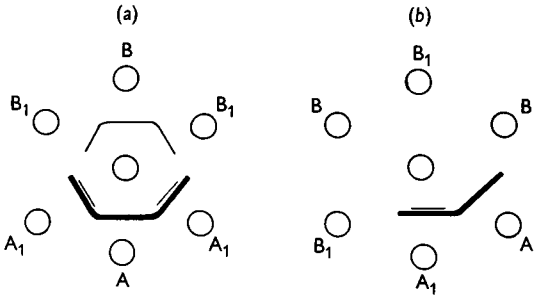


Fig. 3. (*a*) Reaction of goat anti-(rabbit papain-digestion piece I) with chains A and B from whole rabbit γ-globulin and with A and B pieces from papain-digestion piece I (A_1 and B_1). (*b*) Reaction of the same antiserum after absorption with B piece from whole γ-globulin. Experimental details are given in the text.

Table 3. *Amino acid analyses of A piece, B piece and papain-digestion piece II of γ-globulin*

The results for the A and B pieces were calculated from the data of Crumpton & Wilkinson (1963), and those for papain-digestion piece II from the data of Porter (1959), by assuming the molecular weights of the A piece, B piece and piece II to be 22 000, 20 000 and 42 000 respectively (Pain, 1963).

	Amino acid content (moles/mole)			
Amino acid	A piece	B piece	A piece + B piece	Piece II
Lys	7·2	7·5	14·7	16
His	1·7	1·6	3·3	4
Arg	5·4	3·0	8·4	9
Asp	12	16	28	29
Thr	27	25	52	55
Ser	25	19	44	46
Glu	12	18	30	30
Pro	15	10	25	25
Gly	18	15	33	34
Ala	12	13	25	29
Val	19	18	37	42
Met	1·3	0·9	2·2	3·4
Ileu	4·6	5·9	10·5	11
Leu	14	11	25	26
Tyr	7·7	9·6	17·3	17
Phe	5·4	5·4	10·8	13
Cys	4·3	5·5	9·8	12
$CyS{\cdot}CH_2{\cdot}CO_2H$	1·9	0·9	2·8	0
Try	3·6	1·9	5·5	—

Carbohydrate analysis of papain-digestion pieces. Porter (1959) reported that papain-digestion piece III of rabbit γ-globulin contains about two-thirds of the total carbohydrate, piece I about one-third and piece II little or none. This was confirmed and it was assumed that the carbohydrate present in papain-digestion piece I must be in the A piece. However, after isolation of the A and B pieces no carbohydrate could be found in either. It was then found that, when unreduced papain-digestion piece I was run down a Sephadex G-75 column in N-propionic acid, the carbohydrate separated from the protein and appeared as a distinct much-slower-moving peak (Fig. 4). It appears that the carbohydrate of papain-digestion piece I is not covalently bound and is dissociated in N-propionic acid, in contrast with the carbohydrate in papain-digestion piece III and in whole chain A, which is not dissociated by propionic acid. The carbohydrate separated from papain-digestion piece I contained some amino acids but no detailed investigation of the peptide material has been made. The carbohydrate analyses of papain-digestion piece III and the glycopeptide are compared in Table 4. The differences are significant. In both cases paper chromatography of an acid hydrolysate showed again that the hexose consisted of galactose and mannose, together with a trace of fucose.

Assay of antibody activity in the peptide chains. Neither chain A nor chain B prepared from horse γ-globulin or from rabbit γ-globulin, which contained antibody, would precipitate with its specific antigen. When tested qualitatively chain A caused a marked delay in the time of precipitation if added to antiserum before the addition of antigen. This effect was specific. The addition of chain B had no effect at any concentration tested. A direct specific effect of chain A but not of chain B was apparent when tested by the polarization of fluorescence.

Since *N*-terminal amino acid data suggested that chain A might contain as much as 3% of chain B and since this could be due to the presence of 10% of undissociated AB molecules, it was considered essential to obtain quantitative measurements on the antibody activities in the respective chains. Quantitative precipitin reactions were therefore carried out to compare the effects of additions of chains A and B and of whole antibody γ-globulin to antiserum and antigen in optimum amounts. With chain A prepared from rabbit antibody, but not with chain B, there was a specific increase in the weight of precipitate formed, though with a marked delay in the time of precipitation. The effect, however, was small, being only 10–20% more than the control. Studies on the polarization of fluorescence, however, had shown that there was considerably more activity in chain A prepared from horse antibody. Two such preparations were available, namely horse anti-(rabbit γ-globulin) serum and horse anti-(diphtheria toxoid) serum. Different results were obtained with these two preparations (Figs. 5, 6). With horse anti-(rabbit γ-globulin) serum, the addition of horse γ-globulin containing antibody or chain A prepared from it caused an increase in the weight of precipitate formed, although the increase, expressed as a percentage of the weight of antibody added, fell with increasing amounts. This is the way in which a rabbit antiserum behaves and shows that there is no marked inhibition of precipitation in excess of antibody. When added in equivalent amounts, chain A was 70–80% as effective as whole γ-globulin and the effect was specific (Fig. 5); chain A from anti-(diphtheria toxoid) γ-globulin or chain A from inert γ-globulin caused no increase in the weight of the precipitate formed. Chain B from any source was always inactive at all concentrations tested.

With the antitoxin system there was obvious inhibition of precipitation in excess of antibody, as would be expected for a flocculating system. Here chain A appeared to be 100% as effective on a weight basis, or 75% on a molar basis, as the whole

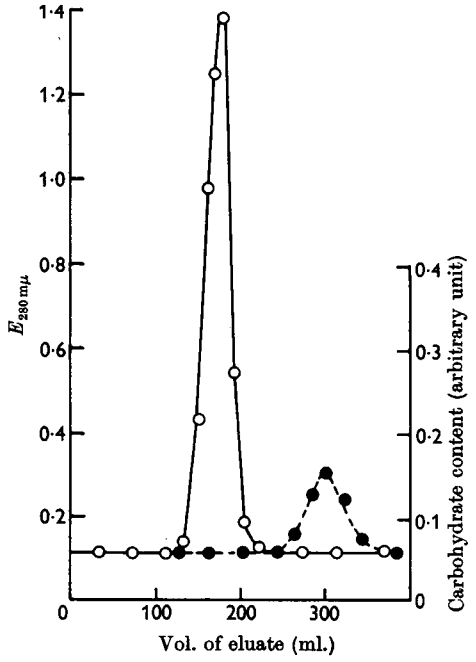


Fig. 4. Separation of glycopeptide from papain-digestion piece I. Carbohydrate was determined by the orcinol method. Piece I was dissolved in N-propionic acid and run through a Sephadex G-75 column (61 cm. × 2·8 cm.) in the same solvent. ○, Extinction at 280 mμ; ●, carbohydrate. Experimental details are given in the text.

Table 4. *Carbohydrate contents of papain-digestion piece III and glycopeptide of γ-globulin*

Experimental details are given in the text. The molecular weight of the papain-digestion piece III was taken to be 50 000. The molecular weight of the glycopeptide is not known, but as it is recovered associated with papain-digestion piece I, the carbohydrate composition of the glycopeptide is expressed as moles/mole of piece I.

Carbohydrate	Carbohydrate content (moles/mole)	
	Piece III	Glycopeptide
Hexose	6·75	3·1
Hexosamine	6·6	2·3
Sialic acid	0·4	0·65
Fucose	0·5	0·2

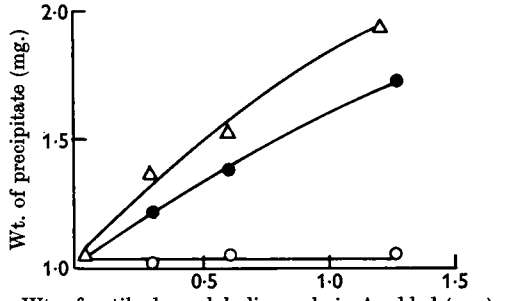


Fig. 5. Co-precipitation in the horse anti-(rabbit γ-globulin)–rabbit γ-globulin system. △, Addition of horse γ-globulin containing antibody to rabbit γ-globulin; ●, addition of chain A from the same preparation of horse γ-globulin containing antibody to rabbit γ-globulin; ○, addition of chain A from horse γ-globulin containing antibody to diphtheria toxoid. The weights of antibody and of antibody chain A added were calculated from the known antibody content of the horse-γ-globulin preparations. Experimental details are given in the text.

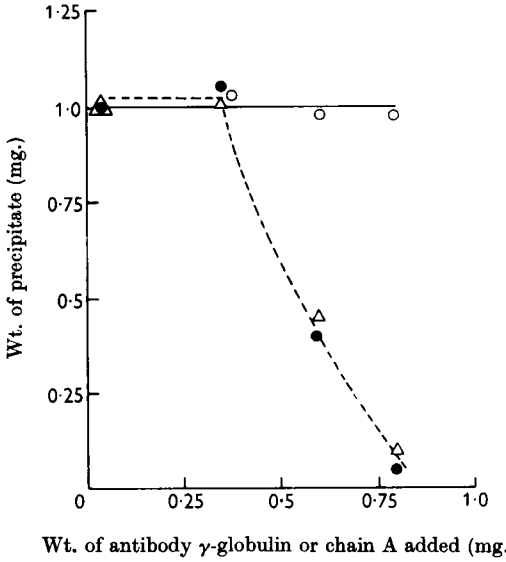


Fig. 6. Inhibition of precipitation in the horse anti-(diphtheria toxoid)–diphtheria toxoid system. ●, Addition of horse γ-globulin containing antibody to diphtheria toxoid; △, addition of chain A from the same preparation of horse γ-globulin containing antibody to diphtheria toxoid; ○, addition of chain A from horse γ-globulin containing antibody to rabbit γ-globulin. The weights of antibody and of antibody chain A added were calculated from known antibody content of the horse-γ-globulin preparations. Experimental details are given in the text.

γ-globulin (Fig. 6). Chain A from anti-(rabbit γ-globulin) or inert γ-globulin was inactive. Chain B from any source was also inactive. There seems little doubt therefore that in horse γ-globulin the specific antibody-combining site is associated with chain A.

DISCUSSION

Evidence has been presented that the reduction of five disulphide bonds/molecule by mercaptoethanol and treatment with N-acetic acid or N-propionic acid causes horse, human and rabbit γ-globulins to dissociate into components of molecular weights of about 20 000 and 50 000. Attempts to achieve dissociation without reduction by treatment with 6 M-guanidine hydrochloride in N-propionic acid at pH 2·8 failed. As these conditions would be expected to split all hydrogen bonds and electrostatic bonds, it is probable that the four components are held together by the disulphide bonds. Cecil & Wake (1962) have shown that interchain bonds are much more easily reduced than intrachain bonds and it is therefore probable, though not certain, that all five disulphide bonds are interchain. The evidence that components A and B are the peptide chains of γ-globulin is: (1) neither full reduction in urea nor conditions that break ester-like bonds in collagen cause further splitting; (2) the molecular weights of the two components are in proportion to the yields obtained and the assumption of two chains A and two chains B gives satisfactory agreement with the molecular weight of the whole molecule; (3) the amino acid content, the carbohydrate content and the *N*-terminal amino acid content of rabbit γ-globulin are accounted for in terms of these two components. However, there is still a possibility that either chain A or chain B consists of two peptide chains held together by an unrecognized type of interchain bond.

The analytical results indicate that chains A and B have been obtained in a purity of at least 95% and support the diagrammatic structure shown in Scheme 1. Correlation of chains A and B with the pieces derived from papain digestion (Fleischman *et al.* 1962) shows the enzymic split to have occurred as indicated. The application of the technique of partial reduction to human γ-globulin showed that the 'S fraction' (equivalent to papain-digestion pieces I and II) contained chain B and the 'F fraction' (equivalent to papain-digestion piece III) contained chain A (Olins & Edelman, 1962; Cohen, 1963). However, direct evidence that the S fraction also contained chain A by reaction with antiserum specific for chain A was not obtained, presumably because of the poor antigenicity of the part of chain A contained in the S fragment (Cohen, 1963). Olins & Edelman (1962) obtained precipitation lines between anti-(S fraction) and chain A but considered this to be due to contamination with chain B. Our suggested distribution of the chains in the enzymic pieces has been substantiated by the chemical and immunological characterization of the reduction products of papain-digestion pieces I and II. The properties of the A and B pieces correlate with those of the whole piece, and the properties of B piece with those of chain B from reduced whole γ-globulin. Analysis of A piece and B piece shows there to be one SH group/molecule of B piece, and two/molecule of A piece. In Scheme 1 three of the disulphide bonds/molecule have been given the most likely positions from this evidence and the remaining two disulphide bonds/molecule reduced by mercaptoethanol allotted to that part of chain A contained in papain-digestion piece III. If chain B is held to chain A and also chain A to chain A by disulphide bonds, other allocations would be difficult but no direct proof of the suggested positions has been obtained.

The carbohydrate analysis shows that at least 95% of the carbohydrate is attached to chain A. Two-thirds of this is in the part of chain A contained in papain-digestion piece III but one-third

is released as glycopeptide on papain hydrolysis. The polysaccharide could have been split by papain but from the known specificity of the enzyme this is unlikely. It seems more probable that the polysaccharide is in two distinct parts, the larger attached to chain A contained in papain-digestion piece III and the smaller to a point in chain A which is fragmented in papain digestion, and that this gives rise to the glycopeptide. There is the further possibility that the original γ-globulin contained a glycoprotein impurity that gave rise to the glycopeptide. However, the hexosamine content of γ-globulins of different electrophoretic mobilities was constant in our preparation of rabbit γ-globulin (as has been reported for human γ-globulin by Heide, Haupt & Schmidtberger, 1959). As the glycopeptide accounts for 25% of the total hexosamine, the impurity would have to show the same electrophoretic distribution as γ-globulin. Hence it is probable that the polysaccharide is in two distinct pieces attached to different parts of the peptide chain, though this is contrary to the view of Rosevear & Smith (1961) and Nolan & Smith (1962), who found the carbohydrate of human and rabbit γ-globulin to be attached to a unique peptide. However, Franklin & Dische (1962) found a higher sialic acid:fucose ratio in the carbohydrate of one papain-digestion piece of human γ-globulin relative to the other and on this basis postulated the presence of two different polysaccharides in human γ-globulin. These conflicting results might be explicable in terms of the known heterogeneity of γ-globulin, which could extend to the carbohydrate moiety as well as to the peptide chains.

The evidence that the antibody-combining site is associated with chain A is not entirely satisfactory, as quantitative measurement depends on co-precipitation or inhibition of precipitation, but the recovery of activity in these terms is so high relative to the immune γ-globulin from which chain A was prepared as to make it most probable that this allocation is correct. Edelman, Benacerraf, Ovary & Poulik (1961) found that, when reduced specific guinea-pig antibody was subjected to electrophoresis on starch gel in 8M-urea and formate buffer, the fraction that is equivalent to our chain B gave distinct lines rather than the broad smudge found with chain B from inert γ-globulin. Further, there was close correlation between the type of banding and type of antibody used. On these grounds it was suggested that chain B contained wholly or partly the antibody-combining site. We have never found banding in chain B from horse or rabbit antibodies under these conditions. At a higher pH there is a striking banding of chain B, but this is common to all γ-globulin chains B. Our evidence suggests that chain B from horse antibody is not concerned in the antibody-binding site, but we have no evidence as to the significance of the obvious complexity of chain B.

If the antibody-combining site is associated with chain A of horse antibody, it must be in that part of chain A found in papain-digestion pieces I and II. It remains to be investigated whether this is in fact correct.

SUMMARY

1. The proposed structure, of four peptide chains with five interchain disulphide bonds, of γ-globulin has been tested by isolation and characterization of the constituent chains.

2. The amino acids, carbohydrate and *N*-terminal amino acids of rabbit γ-globulin have been accounted for in terms of the chains.

3. The papain-digestion pieces have been correlated with peptide chains and they have themselves been dissociated into their respective chains. These have in turn been analysed and characterized.

4. The carbohydrate associated with papain-digestion piece I has been shown to be due to non-covalently bound glycopeptide, and analysis suggested that the carbohydrate of rabbit γ-globulin is present on the molecule in two parts.

5. The antibody-combining site of horse antibody is associated with chain A.

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REFERENCES

Blumenfeld, O. O. & Gallop, P. M. (1962). *Biochemistry*, **1**, 947.
Cecil, R. & Wake, R. G. (1962). *Biochem. J.* **82**, 401.
Cohen, S. (1963). *Nature, Lond.*, **197**, 253.
Crumpton, M. J. & Wilkinson, J. M. (1963). *Biochem. J.* **88**, 228.
Dandliker, W. B. & Feigen, G. A. (1961). *Biochem. biophys. Res. Commun.* **5**, 299.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Edelman, G. M. (1959). *J. Amer. chem. Soc.* **81**, 3155.
Edelman, G. M., Benacerraf, B., Ovary, Z. & Poulik, M. D. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1751.
Edelman, G. M. & Poulik, M. D. (1961). *J. exp. Med.* **113**, 861.
Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.*, Suppl. no. 1, 174.
Franěk, F. (1961). *Biochem. biophys. Res. Commun.* **4**, 28.
Franklin, E. C. & Dische, Z. (1962). *Fed. Proc.* **21**, 33.

Heide, K., Haupt, H. & Schmidtberger, R. (1959). *Behringwerk-Mitteilungen*, **37**, 29.
Johansen, P. G., Marshall, R. D. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Kabat, E. A. & Mayer, M. M. (1961). *Experimental Immunochemistry*, 2nd ed., p. 22. Springfield, Ill.: C. C. Thomas.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Nolan, C. & Smith, E. L. (1962). *J. biol. Chem.* **237**, 446.
Olins, D. E. & Edelman, G. M. (1962). *J. exp. Med.* **116**, 635.
Pain, R. H. (1963). *Biochem. J.* **88**, 234.
Palmer, J. L., Mandy, W. J. & Nisonoff, A. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 49.
Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Porter, R. R. (1959). *Biochem. J.* **73**, 119.
Porter, R. R. (1960). In *The Plasma Proteins*, vol. 1, p. 241. Ed. by Putnam, F. W. New York: Academic Press Inc.
Porter, R. R. (1962). In *Symposium on Basic Problems in Neoplastic Disease*, p. 177. Ed. by Gellhorn, A. & Hirschberg, E. Columbia University Press.
Press, E. M. & Porter, R. R. (1962). *Biochem. J.* **83**, 172.
Ramel, A., Stellwagen, E. & Schachman, H. K. (1961). *Fed. Proc.* **20**, 387.
Rosevear, J. W. & Smith, E. L. (1961). *J. biol. Chem.* **236**, 425.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Stelos, P., Radzimski, G. & Pressman, D. (1962). *J. Immunol.* **88**, 572.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.

Biochem. J. (1963) **88**, 228

Amino Acid Compositions of Human and Rabbit γ-Globulins and of the Fragments Produced by Reduction

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Analyses of amino acid compositions have been reported for normal human γ-globulin (Brand, 1946; Heimer, Woods & Engle, 1962) and for several purified rabbit antibodies (Smith, McFadden Stockell & Buettner-Janusch, 1955; Askonas, Farthing & Humphrey, 1960; Fleischer *et al.* 1961). Similar values were obtained for rabbit antibodies to pneumococcal capsular polysaccharides, anti-(pneumococcal polysaccharide) antibodies present in different electrophoretic fractions of rabbit γ-globulin, and rabbit antibodies to bovine γ-globulin coupled either to a basic or an acidic hapten. Differences between analyses of human γ-globulin and between those of different rabbit antibodies are probably not significant and in all probability are due to variations in experimental technique. Waldschmidt-Leitz, Bretzel & Keller (1960) have reported that γ-globulins from rabbit and human sera contain appreciable amounts of hydroxylysine, 0·60 and 0·85% of the total nitrogen respectively; Askonas *et al.* (1960) have also detected hydroxylysine (0·40% of the total nitrogen) in a fraction of anti-(pneumococcal polysaccharide) antibodies produced in rabbits and separated by electrophoresis. In contrast with these reports, hydroxylysine has apparently not been detected in other Laboratories.

Human and rabbit γ-globulins have been shown (Cohen, 1963; Fleischman, Porter & Press, 1963) to be made up of four peptide chains/molecule. Reduction of γ-globulin under mild conditions gives rise to two types of chains, called A and B, which can be separated in propionic acid. Similarly, reduction of piece II (Porter, 1959) formed by papain digestion of rabbit γ-globulin gives rise to two peptide chains, called A and B pieces.

The results of amino acid analyses of human and rabbit γ-globulins and their constituent peptide chains are presented below, together with the results of an examination of human and rabbit γ-globulins for the presence of hydroxylysine.

MATERIALS AND METHODS

Materials

Rabbit γ-globulin and fragments of rabbit γ-globulin were those described by Fleischman *et al.* (1963). Human γ-globulin and the human-γ-globulin chains A and B were prepared by Cohen (1963); a sample of human γ-globulin (fraction G4; batch no. EG 137) that was kindly supplied by Dr R. A. Kekwick, The Lister Institute, London, S.W. 1, was also used.

Cysteic acid and DL-δ-hydroxylysine hydrochloride were purchased from L. Light and Co. Ltd., L-norleucine and L-2-amino-3-guanidinopropionic acid from California Corp. for Biochemical Research, Los Angeles, and DL-tryptophan and L-tyrosine from Roche Products Ltd. DL-*S*-Carboxymethylcysteine was synthesized by the method of Michaelis & Schubert (1934) and was recrystallized three times from