Biochem. J. (1968) **107**, 559
Printed in Great Britain

Comparative Studies of Two Types of Bovine Immunoglobulin G Heavy Chains

BY C. P. MILSTEIN AND A. FEINSTEIN
Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 18 *October* 1967)

'Fingerprints' of bovine colostrum and serum immunoglobulin G1 heavy chains were extremely similar, but different from serum immunoglobin G2 heavy chains. Serum immunoglobulin G1 and immunoglobulin G2 heavy chains were treated with cyanogen bromide. The fractions from the *C*-terminal end of the heavy chains were isolated and the amino acid sequence of this fraction from immunoglobulin G2 was:

His-Glx-Ala-Leu-His-Asx-His-Tyr-Met-Gln-Lys-Ser-Thr-Ser-Lys-Ser-Ala-Gly

The amino acid composition of this fraction from immunoglobulin G1 was the same except for the methionine, which in immunoglobulin G1 was replaced by threonine.

It has been shown (Pierce & Feinstein, 1965) that γ-globulin components of two distinct antigenic types can be separated from bovine serum. Further examination (A. Feinstein & A. E. Pierce, unpublished work) has established that the antigenic and electrophoretic differences reside in the heavy (γ) polypeptide chains. The antigenically identical components earlier referred to as S1 and S2 will now be referred to as IgG1,* and the antigenically different component S3 will be denoted by IgG2. IgG1 is selectively concentrated in bovine colostrum. Precipitating antibody appears in both fractions, but, whereas IgG1 antibody fixes guinea-pig complement and sensitizes bovine skin, neither activity has been demonstrated in IgG2 preparations. It would clearly be of considerable interest to compare the primary structure of the IgG1 and IgG2 heavy chains; such studies would have a twofold interest. They should throw light on the origin of these two related heavy chains, and it might be possible to identify those stretches in the sequence responsible for the functional differences of the IgG1 and IgG2 antibodies.

Givol & Porter (1965) have reported methods for the isolation of *C*-terminal peptides of γ-chains of IgG. In the present paper these methods have been applied to the γ-chains of bovine IgG1 and IgG2 immunoglobulins.

MATERIALS AND METHODS

Immunoglobulins IgG1 and IgG2. These were isolated from bovine sera and colostrum (about 100ml.), by using a Whatman DEAE-cellulose 50 column (64cm.×4cm.) equilibrated with 0·01M-Na_2HPO_4, on which the IgG2 component was unadsorbed. Subsequently a buffer gradient was established, by using 0·3M-NaH_2PO_4, to elute the IgG1 component; the gradient was produced in a closed 2l. mixing vessel. For even larger-scale fractionations (500ml. of serum) a stepwise elution instead of the gradient was used. In these cases the DEAE-cellulose column (90cm.× 8cm.) was equilibrated as above, but after the elution of the IgG2 component, instead of the application of a gradient, 0·044M-phosphate buffer, pH6·97 (6 vol. of 0·044M-Na_2HPO_4+4vol. of 0·044M-NaH_2PO_4), was run through the column to elute the IgG1 component. Fractions were pooled on the basis of their immunoelectrophoretic patterns. An IgG2 preparation (Armour IgG2) was similarly obtained starting from a commercial bovine γ-globulin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex).

The light and heavy chains were separated on Sephadex G-75 gel with N-propionic acid by the method of Fleischman, Pain & Porter (1962). Fractions were tested by immunoelectrophoresis, and pools of heavy chain used for 'fingerprint' analysis were made from fractions showing the lowest contamination with light chain.

IgG1 heavy chains were isolated from two different individual bovine sera, and IgG2 heavy chains were isolated from only one of these sera and from Armour IgG2.

Oxidation of heavy (and light) chains. Samples (10mg.) of freeze-dried heavy chains (or light chains) were dissolved in 0·4ml. of 98% (w/w) formic acid, 0·4ml. of performic acid was added to each, and the mixtures were allowed to react at room temperature for 30min. and were then dried in a desiccator.

Tryptic digests. These, unless otherwise stated, were done in 0·75% NH_4HCO_3 solution, pH7·9, at 37° for 18hr. The enzyme/substrate ratio used was 1:100 (w/w) and the final concentration of protein was 2·5mg./ml. Trypsin was a twice-crystallized salt-free preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A. (batch TR3F 6401).

* Abbreviations: IgG, immunoglobulin; DNS, 1-dimethylaminonaphthalene-5-sulphonyl; Asx and Glx (in amino acid sequences), aspartic acid or asparagine and glutamic acid or glutamine respectively.

High-voltage paper electrophoresis. This was carried out in an apparatus similar to that described by Michl (1951). The buffer systems and coolants at pH 6·5, 3·5 and 2·0 were as described by Ambler (1963). The voltage used was 53 v/cm.

'*Fingerprints*'. These were carried out on tryptic digests of oxidized heavy chains (or light chains) that had been dried two or three times under vacuum in the presence of P_2O_5, to evaporate the NH_4HCO_3. The dried digest was dissolved in water and a 1 mg. sample was applied as a band (1·3 cm. long) to a sheet of Whatman 3MM paper. The sample was run by high-voltage paper electrophoresis at pH 6·5 at 53 v/cm. for 1 hr. The strip containing the sample was cut out and sewn to a new sheet of paper, and the sample was run at right angles by descending chromatography in butan - 1 - ol – acetic acid – water – pyridine (15:3:12:10, by vol.) for 15 hr.

Identification and location of peptides in paper. Most peptides were located by dipping the paper in 0·25% (w/v) ninhydrin solution in acetone and leaving them in the dark for at least 12 hr. or heating them at 80–100° for 5 min. after dipping. The specific colour tests for peptides containing tyrosine (Jepson & Smith, 1953) and histidine (Dent, 1947) were also used. Mobilities of peptides at pH 3·5 were calculated by taking the mobility of free histidine during the same run as 1.

Cyanogen bromide cleavage. A preliminary experiment was carried out with 65 mg. of Armour bovine serum IgG2 heavy chains. On the basis of this experiment, a larger-scale cleavage was then performed. A sample (387 mg.) of bovine serum IgG1 heavy chain was dissolved in 9·7 ml. of 70% (w/v) formic acid, 1·55 g. of CNBr was added and the mixture was incubated for 24 hr. at room temperature. The reaction mixture was diluted with water and freeze-dried.

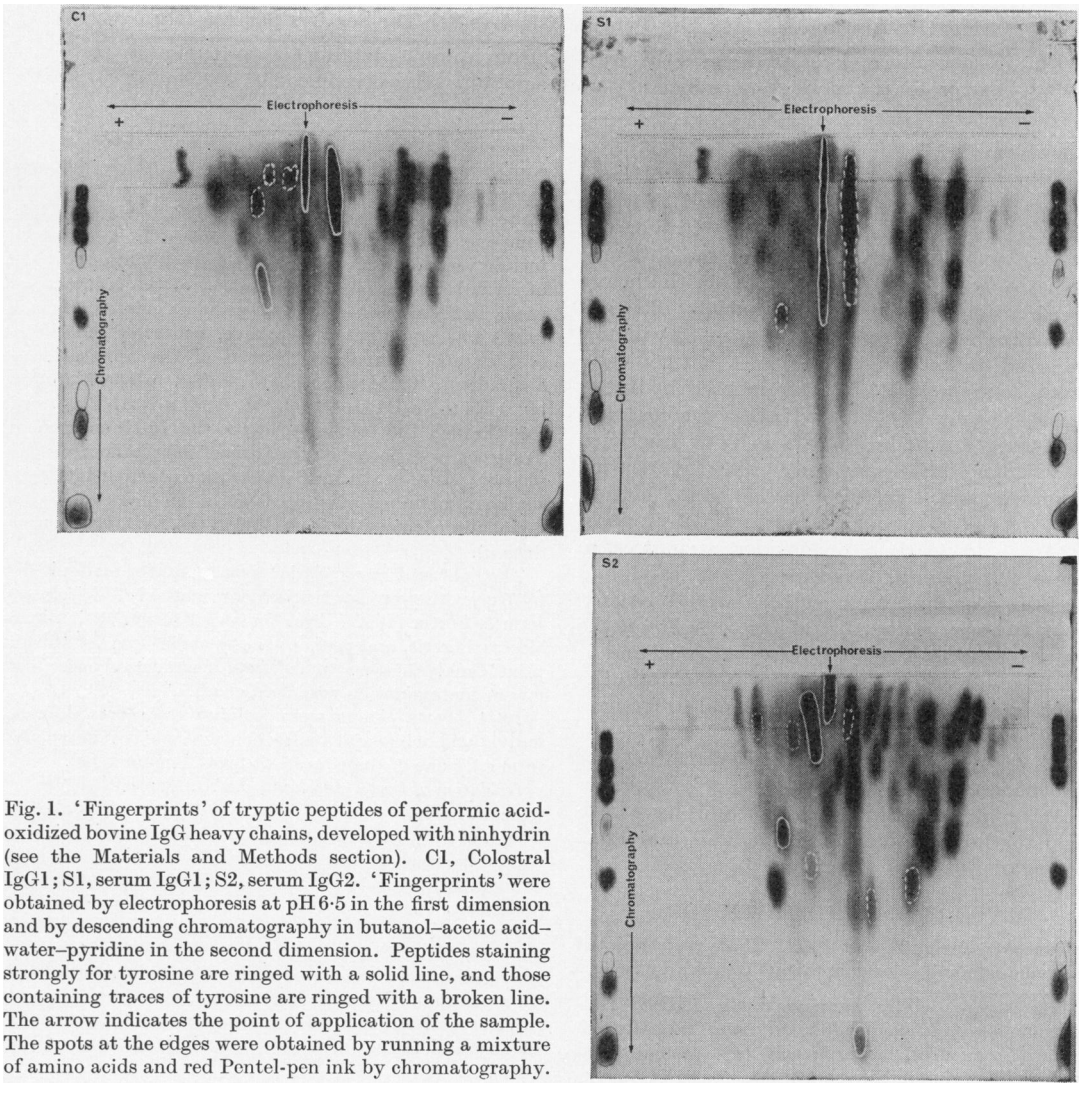


Fig. 1. 'Fingerprints' of tryptic peptides of performic acid-oxidized bovine IgG heavy chains, developed with ninhydrin (see the Materials and Methods section). C1, Colostral IgG1; S1, serum IgG1; S2, serum IgG2. 'Fingerprints' were obtained by electrophoresis at pH 6·5 in the first dimension and by descending chromatography in butanol–acetic acid–water–pyridine in the second dimension. Peptides staining strongly for tyrosine are ringed with a solid line, and those containing traces of tyrosine are ringed with a broken line. The arrow indicates the point of application of the sample. The spots at the edges were obtained by running a mixture of amino acids and red Pentel-pen ink by chromatography.

The CNBr digest was then fractionated on a Sephadex G-100 column (125 cm. × 2·5 cm.) and the fractions were further purified on a Sephadex G-50 column (135 cm. × 2 cm.) in 0·1 N-NH_3 soln., followed by paper electrophoresis at pH 3·5 (Press, Piggot & Porter, 1966). A sample (303 mg.) of bovine serum IgG2 heavy chain was subjected to the same treatment. However, the size of the Sephadex G-100 column used in this case was 89 cm. × 4 cm., and it was found to work better than the one used for the CNBr-treated IgG1 heavy chain, so much so that it was not necessary to re-run through Sephadex G-50 the last fraction eluted from Sephadex G-100; this last fraction was run directly on paper electrophoresis at pH 3·5. In both cases (IgG1 and IgG2) the elution patterns given by the columns were followed by measurement of the extinction at 280 mμ, and the last fraction to be eluted, which had little or no absorption at 280 mμ, was detected by spotting samples of 50 μl. out of every other tube on to paper and staining for the histidine reaction.

Total acid hydrolysis. Peptide samples (0·05–0·1 μmole) were hydrolysed in 50 μl. of constant-boiling HCl in evacuated sealed tubes at 105° for 20 hr., unless otherwise stated.

Amino acid composition. The amino acid compositions of very simple peptides and of peptides obtained only in very small amounts were determined by paper electrophoresis; the amino acids released by total acid hydrolysis were separated by electrophoresis at pH 2·0 on Whatman no. 1 paper for 15–20 min. at 105 v/cm. Quantitative amino acid analyses were performed in a Technicon amino acid analyser by the method described by Wright (1967), on a 125 cm. × 0·6 cm. column.

N-Terminal analyses. These were performed by the 'dansyl' method described by Gray (1967), but the DNS-amino acids were separated on polyamide layer chromatography as described by Woods & Wang (1967), with their solvents 1 and 2 for first and second dimensions respectively. Solvent 1 was prepared by using 98% (w/w) formic acid instead of 90%. These two systems did not give good separation of DNS-serine from DNS-threonine or of DNS-glutamic acid from DNS-aspartic acid. In these cases the polyamide layer was re-run in the second dimension (S. Magnusson, personal communication) with Crowshaw, Jessup & Ramwell's (1967) systems IV and VII. System IV, ethyl acetate–methanol–acetic acid (20:1:1, by vol.), separated threonine from serine, and system VII, methyl acetate–35% (w/v) NH_3 soln. (19:1, v/v), resolved glutamic acid and aspartic acid. Every sample was run together with standards prepared as described by Boulton & Bush (1964).

Edman degradation. The procedure described by Gray (1967) in the section entitled 'Sequential Degradation plus Dansylation' was followed. The DNS-amino acids were identified by polyamide layer chromatography as described above.

RESULTS

'Fingerprints' of heavy chains of bovine serum and colostrum IgG1 looked extremely similar when stained with ninhydrin, but the number of tyrosine-positive spots was smaller in the serum IgG1, probably owing to a slight difference in the amounts used (Fig. 1). This similarity was already obvious after the first-dimension run. Fig. 1 also shows the differences between heavy chains of serum IgG2 and heavy chains of serum or colostrum IgG1. One outstanding difference was the presence, in the pattern given by the heavy chain of serum IgG2, of two strong spots highly basic at pH 6·5 with slow mobility in butanol–acetic acid–water–pyridine. There was also one acidic spot with high mobility in the chromatography solvent. Another outstanding difference was the presence in the patterns of IgG1 heavy chains of a spot highly acidic at pH 6·5 that hardly moved in the chromatography system used. The two serum IgG1 heavy chains gave identical 'fingerprints', as did the two IgG2 preparations. Reference to the 'fingerprint' of bovine light chain (Fig. 2) shows that these characteristic differences could not have been due to differences in contamination with light-chain peptides, since strong spots are absent from these regions in the light-chain pattern.

Heavy chains of serum IgG1 were treated with cyanogen bromide, as described in the Materials and Methods section. The yield of the last fraction from the Sephadex G-50 column was subsequently seen to be 83% (by wt.) of the theoretical yield from the heavy chain used. This fraction was run by paper

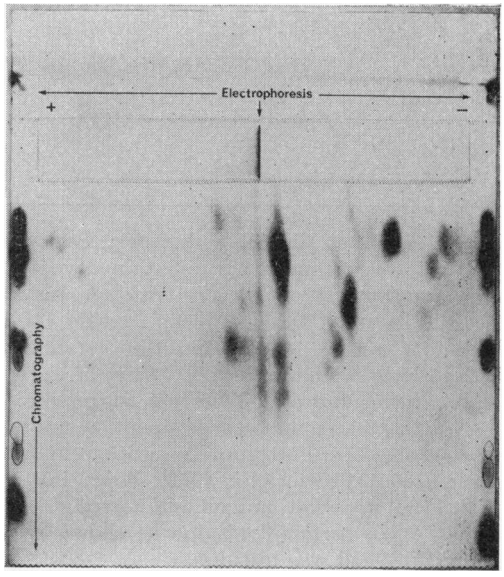


Fig. 2. 'Fingerprint' of a tryptic digest of performic acid-oxidized bovine IgG light chain, obtained by electrophoresis at pH 6·5 in the first dimension and by descending chromatography in butanol–acetic acid–water–pyridine in the second dimension, developed with ninhydrin (see the Materials and Methods section). The arrow indicates the point of application of the sample. The spots at the edges were obtained by running a mixture of amino acids and red Pentel-pen ink by chromatography.

Table 1. *Amino acid composition of peptides 1M, 2M1 and 2M2*

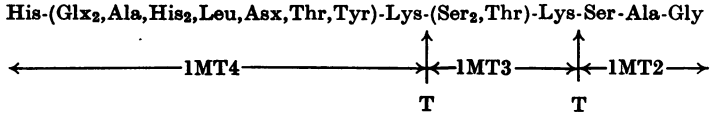
The quantitative analyses were kindly performed by Dr J. L. Mangan with the Technicon amino acid analyser. Values for serine and threonine were not corrected for decomposition during hydrolysis. Values for homoserine and homoserine lactone were calculated from the analyses of a homoserine standard before and after hydrolysis. The standard was hydrolysed under the same conditions as the sample.

	Amino acid content (mole/mole)		
	Peptide 1M	Peptide 2M1	Peptide 2M2
Asp	1·1	1·0	—
Thr	1·9	<0·1	1·1
Ser	2·8	0·1	3·0
Glu	1·9	1·1	1·0
Gly	1·1	<0·1	1·0
Ala	1·8	1·1	1·0
Leu	1·0	0·9	—
Tyr	1·0	1·0	—
Lys	2·1	0·1	1·8
His	3·0	2·9	—
Homoserine + homoserine lactone	—	0·9	—
Total residues	17·7	9·3	8·9

band (band 1MT3) had a mobility of 0·69. The histidine reagent showed a third band (band 1MT4) running between peptides 1MT2 and 1MT3 with a mobility of 0·53. The fact that this peptide (band 1MT4) was not stained by ninhydrin might have been due either to the small amount present or to the presence of histidine as *N*-terminus. The peptide 1MT3 was purified by paper electrophoresis at pH 6·5. The amino acid compositions of peptides 1MT2 and 1MT3 were determined by paper electrophoresis at pH 2·0 after total acid hydrolysis and were: peptide 1MT2, Ser (+), Ala (+), Gly (+); peptide 1MT3, Lys (+), Ser (++), Thr (+) [(+) gives the intensity of the ninhydrin reaction on paper; these values are only approximate]. The amino acid composition of peptide 1MT4 was not determined owing to lack of material. The *N*-terminal residue of peptide 1MT2 was serine and its sequence, determined by the combined Edman degradation and 'dansyl' technique, was:

Ser-Ala-Gly

The fact that this peptide did not contain lysine suggested that it came from the *C*-terminus. Peptides 1MT3 and 1MT4 should have had lysine as their *C*-terminal residue because of trypsin specificity and therefore the partial sequence of peptide 1M was likely to be:



electrophoresis at pH 3·5, and a very basic band (band 1M) was obtained that was stained weakly by ninhydrin but gave a very intense histidine reaction; its mobility was 0·69. Peptide 1M was eluted; its amino acid composition is shown in Table 1. It was found to contain 18 residues present in molar ratios, but no homoserine, suggesting that this was the *C*-terminal octadecapeptide similar to those reported by Porter and co-workers (Givol & Porter, 1965; Press *et al.* 1966; Weir, Porter & Givol, 1966) in rabbit, human and horse IgG heavy chains. Its *N*-terminal residue as shown by the 'dansyl' method was histidine.

Since peptide 1M contained two lysine residues (Table 1) susceptible to tryptic action, about 100 mμmoles of it were digested with 50 μg. of trypsin in 1% (w/v) ammonium hydrogen carbonate, pH 7·95, for 24 hr. at 37°. The tryptic digest was fractionated by paper electrophoresis at pH 3·5. On development with ninhydrin two bands appeared; one of these (band 1MT2) ran with an Ala-Gly marker, having a mobility of 0·38, and the other

As described in the Materials and Methods section, the cyanogen bromide-treated heavy chain from serum IgG2 was only run through Sephadex G-100. The last fraction to be eluted, which had a very low extinction at 280 mμ but an intense histidine-positive reaction, was run on paper electrophoresis at pH 3·5. Though the histidine reagent showed one very strong positive band (band 2M1), ninhydrin gave evidence of another very basic band (band 2M2) that gave a stronger ninhydrin reaction than band 2M1 and ran just behind it. The mobilities of these two bands were 0·83 and 0·71 respectively. Peptide 2M2 was purified by re-running it at pH 3·5. Table 1 shows the amino acid composition of both peptides. Peptide 2M1 (Table 1) was composed of only nine amino acid residues, three of which were histidine. It contained homoserine and homoserine lactone, indicating that methionine was present as *C*-terminus (Gross & Witkop, 1961). Therefore peptide 2M1 was not the *C*-terminal peptide of the heavy chain of IgG2. Apart from the methionine, the other eight amino acids were the same as the eight *N*-terminal residues of the *C*-terminal octa-

decapeptide of the heavy chain of rabbit, human and horse IgG reported by Givol & Porter (1965), Press *et al.* (1966), Prahl (1966) and Weir *et al.* (1966). Peptide 2M2 contained nine amino acid residues (Table 1), and it did not contain homoserine or homoserine lactone.

The *N*-terminal residue of peptide 2M1 determined by the 'dansyl' method was histidine. Seven residues of this peptide, including the histidine, were determined by the combined Edman and 'dansyl' method, and since the *C*-terminus must have been methionine the sequence of peptide 2M1 was:

His-Glx-Ala-Leu-His-Asx-His-Tyr-Met

The mobility of peptide 2M2 at pH 6·5 was 0·49. The charge corresponding to a peptide of that mobility and molecular weight is 2 (Offord, 1966), suggesting that the glutamic acid residue was present as glutamine. The sequence of peptide 2M2, which was determined by the combined Edman and 'dansyl' method, was:

Gln-Lys-Ser-Thr-Ser-Lys-Ser-Ala-Gly

DISCUSSION

The 'fingerprint' results suggest that the primary structures of the heavy chains of IgG1 from bovine serum and colostrum are similar and differ from that of serum IgG2. As a result it seems likely that the IgG1 secreted in colostrum has the same primary structure as serum IgG1. It has been suggested that the secretion of colostral immunoglobulin A in human and rabbit (Hong, Pollara & Good, 1966; Cebra & Small, 1967) is facilitated by the addition of a polypeptide chain ('transport piece') not present in the serum protein. However, the elution patterns of the polypeptide chains of the reduced IgG1 proteins showed only heavy chain and light chain, and no difference in molecular weight was detected between serum IgG1 and colostral IgG1 (A. Feinstein, unpublished work). Consequently, it would appear that any selective secretion of IgG1 in colostrum must be accounted for in terms of the structural differences between the heavy chains of IgG1 and IgG2, since identical light chains are present in both immunoglobulins.

The amino acid composition and partial sequences of the *C*-terminal octadecapeptide of IgG1, as well as the sequence obtained by addition of the two nonapeptides of IgG2, resemble very closely the sequence of the *C*-terminal octadecapeptides reported by Givol & Porter (1965), Press *et al.* (1966), Prahl (1966) and Weir *et al.* (1966) for rabbit, human and horse IgG heavy chains. Both of them are more similar to horse IgG than to any of the others published (Table 2). It is remarkable that both bovine heavy chains have alanine in position 2 from the *C*-terminus, since proline occupies this position in all previously reported sequences of IgG subclasses of human, horse and rabbit. This substitution of alanine for proline could have arisen by a single base mutation (guanine for cytosine) apparently before the duplication of the ancestral IgG1 heavy-chain gene. Again, unlike all other reported sequences, both bovine heavy chains have threonine in the sixth position from the *C*-terminus. However, this appears to be a more variable position since human, horse and rabbit IgG have leucine, valine and isoleucine respectively. It is noteworthy that these last three residues could each have arisen from any other of the three by a single base change in the first position of the coding triplet. However, if threonine arose as a single base change from one of these three it could only have been by substituting cytosine for uracil in the second position of the triplet. This suggests that isoleucine may have been the precursor residue.

The single difference between the two bovine heavy chains is the methionine residue at the position 10 from the *C*-terminus in IgG2. As in all other reported sequences, bovine IgG1 has threonine in this position. It is possible therefore that the common ancestor of horse and bovine IgG contained only one heavy chain, the formation of the two heavy-chain types being a more recent event.

The main aim of these studies is to correlate structural differences in heavy chains of IgG1 and

Table 2. *C-Terminal sequences of the heavy chains of several immunoglobulins*

The Table indicates positions where the various immunoglobulins differ from the sequence of human IgG (γ_2b, We), shown at the top. Residue 9 from the *C*-terminus of bovine IgG2 is Gln. It is not known whether residue 9 from the *C*-terminus of bovine IgG1 and residues 13 and 17 from the *C*-terminus of bovine IgG1 and IgG2 are present as Glu or Gln and Asp or Asn.

Species	-Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly-	Reference
Human IgG (γ_2b, We)	—	—	—	—	—	—	—	—	—	—	—	—	—	Leu	—	Leu	—	—	—	Press *et al.* (1966)
Human IgG (γ_2c, Vi)	—	—	—	—	—	—	—	Arg	Phe	—	—	—	—	Leu	—	Leu	—	—	—	Prahl (1966)
Rabbit IgG	—	—	—	—	—	—	—	—	—	—	—	—	—	Ile	—	Arg	—	—	—	Givol & Porter (1965)
Horse IgG	—	—	—	—	—	—	—	—	—	—	—	—	—	Val	—	Lys	—	—	—	Weir *et al.* (1966)
Horse IgG(T)	—	—	—	—	Val	Glu	—	—	—	—	—	—	—	Asn	Val	—	His	—	—	—	Weir *et al.* (1966)
Bovine IgG1	—	—	—	—	—	—	—	—	—	—	—	—	—	Thr	—	Lys	—	Ala	—	Present paper
Bovine IgG2	—	—	—	—	—	—	—	—	—	Met	—	—	—	Thr	—	Lys	—	Ala	—	Present paper

IgG2 with their known functional differences. The 'fingerprint' results indicate that several other substitutions must be present, scattered along the heavy chain.

Mr E. V. Deverson's skilful technical assistance is gratefully acknowledged.

REFERENCES

Ambler, R. P. (1963). *Biochem. J.* **89**, 349.
Boulton, A. A. & Bush, I. E. (1964). *Biochem. J.* **92**, 11P.
Cebra, J. J. & Small, P. A. (1967). *Biochemistry*, **6**, 503.
Crowshaw, K., Jessup, S. J. & Ramwell, P. W. (1967). *Biochem. J.* **103**, 79.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. 1, p. 174.
Givol, D. & Porter, R. R. (1965). *Biochem. J.* **97**, 32C.
Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 139. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Gross, E. & Witkop, B. (1961). *J. Amer. chem. Soc.* **83**, 1510.
Hong, R., Pollara, B. & Good, R. A. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 602.
Jepson, J. B. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.
Michl, H. (1951). *Mh. Chem.* **82**, 489.
Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
Pierce, A. E. & Feinstein, A. (1965). *Immunology*, **8**, 106.
Prahl, J. W. (1966). *Proc. Roy. Soc.* B, **166**, 108.
Press, E. M., Piggot, P. J. & Porter, R. R. (1966). *Biochem. J.* **99**, 356.
Weir, R. C., Porter, R. R. & Givol, D. (1966). *Nature, Lond.*, **212**, 205.
Woods, K. R. & Wang, K. T. (1967). *Biochim. biophys. Acta*, **133**, 369.
Wright, P. C. (1967). *Technicon Monogr. no. 2: Proc. 5th Colloq. Amino Acid Analysis*, p. 164. Domont, France: Technicon International Division.