Structure of Heavy Chain from Strain 13 Guinea Pig Immunoglobulin-G(2). II. Amino Acid Sequence of the Carboxyl-Terminal and Hinge Region Cyanogen Bromide Fragments*

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ABSTRACT: Two CNBr fragments from the heavy (γ_2) chain of IgG(2) isolated from inbred guinea pigs were sequenced. These fragments were the C-terminal octadecapeptide (C-5) and a 65 residue peptide (C-1-c) containing the hinge region. Fragment C-1-c contained three of the four labeled half-cystines found on γ_2 chain prepared by mild reduction and subsequent alkylation with iodoacetate-^{14}C. The amino acid sequences of both fragments were closely homologous with those from corresponding sections of IgG from other species. However, although the proline-rich hinge regions from the guinea pig, rabbit, and human proteins were nearly identical in sequence, the adjacent sections N terminal to the hinge region all varied markedly in amino acid sequence and number of residues present.

The isolation of five fragments, accounting for ~303 amino acid residues, from a CNBr digest of the heavy chain of strain 13 guinea pig IgG(2)[1] has recently been reported (Birshtein *et al.*, 1971a). Our aim is to determine the sequence of as much of the γ_2 chain as is permitted by the occurrence of a single primary structure and thus to define those sections which are "variable" and those which are "constant" within this polymorphic form of heavy chain. The first two CNBr fragments to be isolated from the guinea pig γ_2 chain and sequenced were the one from the carboxyl terminus and the one from the middle which includes the proline-rich hinge region.

The carboxyl-terminal octadecapeptide from CNBr digests, recognized by its lack of homoserine, has previously been sequenced after isolation from normal rabbit, horse, and cow IgGs (Givol and Porter, 1965; Weir *et al.*, 1966; Milstein and Feinstein, 1968) and from human IgG myeloma proteins of various subclasses and allotypes (Press *et al.*, 1966; Prahl, 1967). Various lengths of a section occurring approximately in the middle of γ chain, rich in proline and containing some of the interchain half-cystine residues, have been sequenced using IgG derived from normal rabbit serum (Hill *et al.*, 1967; Cebra, 1967; Smyth and Utsumi, 1967) and from four subclasses of human IgG from myeloma patients (Steiner and Porter, 1967; Frangione *et al.*, 1969). The part of this section having a high proline content and occurring in a position within the IgG molecule which electron micrographs suggest is flexible (Green, 1969) has been called the hinge region.

The amino acid sequences of the C-terminal octadecapeptide and a 65 residue hinge region fragment from guinea pig IgG(2), reported here, indicate extensive conservation among species of the amino acid sequence at the carboxyl terminus and in the proline-rich region of heavy chain, some interchanges so far peculiar to this species, and an impressive variability among species in the section containing interchain half-cystines immediately amino terminal to the hinge region.

Materials and Methods

Preparation of Fragments C-5 and C-1-c from a CNBr Digest of γ_2 Chain. These fragments were prepared as outlined in the preceding paper (Birshtein *et al.*, 1971a). The γ_2 chain used as source material was derived from IgG(2) after mild reduction with dithiothreitol and alkylation of easily reduced half-cystine residues with iodoacetate-^{14}C as described (Birshtein *et al.*, 1971a). Thus C-1-c was always radiolabeled and its isolation could be monitored by measuring radioactivity in small fractions of column effluent in a liquid scintillation counter (Birshtein *et al.*, 1971a). An alternate, single-step procedure for isolation of only C-3, C-1-C, and C-5, in high yield and of purity suitable for obtaining constituent peptides, was often used. The freeze-dried CNBr digest of γ_2 chain (150 mg) was dissolved in 7 M guanidine hydrochloride, 0.1 M in Tris-acetate buffer (pH 8.0, 7.5 ml). The solution was made 0.05 M in dithiothreitol and allowed to stand in a covered container at room temperature for 2 hr. The protein was then alkylated by adding cold iodoacetate, dissolved in 2 M Tris, and the pH of the reaction mixture was maintained at 8.0 with 2 M Tris for 20 min (Birshtein *et al.*, 1971a). The entire reaction mixture was then directly applied to a column of Sephadex G-75 (3.4 × 180 cm) equilibrated with 0.05 M NH_4HCO_3. The effluent was collected in 10-ml fractions and C-1-c and C-5 emerged in 1.9 and 3.1 void volumes, respectively.

Enzymic Digestion of C-5 and C-1-c. These fragments (1–4 μmoles) were dissolved in 0.005 M NH_4HCO_3 buffer (pH 8.0) (5–15 ml), and trypsin or chymotrypsin was added (0.5–2.0 mg). The digestion mixture was incubated at 37° for 8 hr.

Separation of Component Tryptic Peptides of C-5 and C-1-c. Figure 1 shows an elution profile of a tryptic digest of C-5 indicating how peptides T1 (pool III), T2 (pool II), and T3

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[1] The nomenclature is in accord with that suggested in *Bull. W. H. O.* (1964), *30*, 447, and with that proposed by the Conference on Nomenclature for Animal Immunoglobulins, Prague, June 1969.

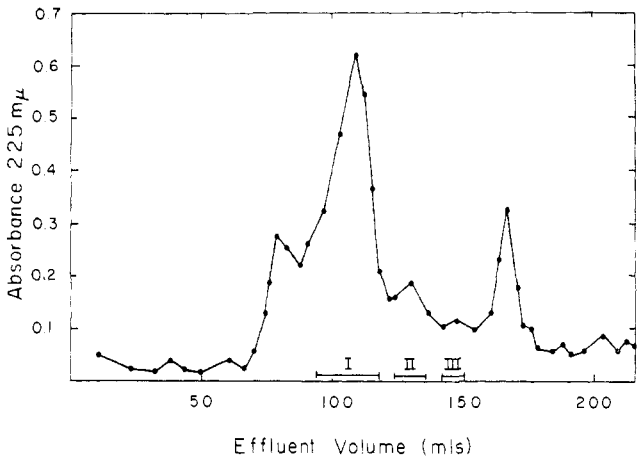

FIGURE 1: Separation of tryptic peptides from C-5. The digest of C-5 (2 μmoles) was applied to a column of Sephadex G-25, fine (1.8 × 82 cm), and elution was carried out with 0.05 M NH_4OH (3.3-ml fractions).

(pool I) can be resolved by gel filtration on a column of Sephadex G-25.

The isolation of the component tryptic peptides of C-1-c is shown in the scheme in Figure 2. First, the tryptic digest was fractionated on a column of Sephadex G-50 (fine) as depicted in Figure 3. Pool II contained the single peptide T28–29, which has three lysine residues and most of the radioactivity. This peptide was a major product from the tryptic digestion of isolated C-1-c. On the other hand, when totally reduced and carboxymethylated γ_2 chain was digested with trypsin, the major products from this section of C-1-c were the separate peptides T28 and T29 (D. C. Benjamin and Q. Z. Hussain, unpublished data). These were isolated from the digest of heavy chain after gel filtration on Sephadex G-50 and subsequent chromatography on Dowex 1-X2 using a Technicon peptide analyzer and procedures to be described (Birshtein *et al.*, 1971b). Pool III (Figure 3) contained peptides T27 and T32. These were separated by applying the mixture (1–2 μmoles) in 0.01 M potassium phosphate buffer (pH 6.3) to a column of Sephadex A-25 (1.0 × 8.0 cm), equilibrated in the same buffer. Peptide T32 was eluted in the void volume while peptide T27 was eluted with 0.1 M potassium phosphate buffer (pH 6.3). Final purification of T27 was achieved by preparative high-voltage paper electrophoresis at pH 3.6. Pool IV, shown in Figure 3, contained peptides T30, T31, and T33. Peptide T33 was separated from the other two by gel filtration of the mixture (1–2 μmoles) on a column of Sephadex G-25 (2.0 × 240 cm) equilibrated with 0.05 M NH_4OH, from which T30 and T31 emerged in 1.2 and T33 in 1.4 void volumes. Finally, peptides T30 and T31 were resolved by preparative paper electrophoresis at pH 3.6.

Enzymes. Trypsin (twice crystallized), α-chymotrypsin (purified), carboxypeptidase A (twice crystallized), carboxypeptidase B, and papain (twice crystallized) were obtained from Worthington Biochemical Corp.

Trypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Cyclo Chemical Corp.) before use (Kostka and Carpenter, 1964). Pepsin was purified by chromatography (Ryle and Porter, 1959) by Dr. A. P. Ryle. Thermolysin (Calbiochem) and pronase (Calbiochem) were used as supplied.

Determination of Amino Acid Sequences. The Gray modification of Edman's procedure for the sequential removal of amino acids was employed (Gray, 1967), and the subtractive method (Konigsberg, 1967) was used in most instances to determine which residue was lost at each step. Occasionally, dansylation was used together with the Edman procedure to mark the new amino-terminal residues (Gray, 1967). The dansyl derivatives of amino acids were identified by chromatography on thin layers of silica gel (Morse and Horecker, 1966; Mesrob and Holeysovsky, 1966). In some instances, particularly in the case of a histidine residue, the phenylthiohydantoin derivatives were identified by thin-layer chromatography (Jeppsson and Sjöquist, 1967). Carboxyl-terminal residues were determined by hydrazinolysis (Bradbury, 1958) or by carboxypeptidase digestion. Carboxypeptidase A was dissolved in 1 M NH_4HCO_3 at a concentration of 1 mg/ml. Ordinarily, 50 μg of enzyme was added to 0.02–0.04 μmole of peptide in 1.0 ml of dilute NH_4HCO_3 (pH 8.1) and digestion was allowed to proceed for 0.5–60 min. The reaction was stopped at the desired time by adding an equal volume of 0.2 M citrate buffer (pH 2.2) and freezing. Similar conditions were used for carboxypeptidase B hydrolysis after addition of 64 μg (4 μl) of enzyme to the substrate solution.

Other Procedures. Volatile buffers were removed from peptides by rotary evaporation or freeze-drying; nonvolatile buffer salts were removed by filtering the peptide solutions through a column of Sephadex G-10 equilibrated with 0.05 M NH_4OH or 0.05 M acetic acid. Preparative high-voltage electrophoresis was carried out on Whatman No. 3MM sheets (46 × 57 cm) under Isopar (Esso) at pH 3.6 or 6.5 using a Savant apparatus and buffers as described (Crumpton and Wilkinson, 1965). Trifluoroacetylation of peptides was done in 5 M guanidine hydrochloride as described previously (Cebra *et al.*, 1968a) except that the substituted peptides were freed of reagents using gel filtration on Sephadex G-10 in 0.1 M ammonium formate followed by freeze-drying. Other procedures and materials used in the experimental work detailed in this paper have been described in the preceding paper (Birshtein *et al.*, 1971a) or elsewhere (Cebra *et al.*, 1968a,b; Birshtein *et al.*, 1971b).

Specific Radioactivity of Peptides. A 0.01–0.03-μmole sample of a labeled peptide was hydrolyzed as described (Birshtein *et al.*, 1971a). After removal of HCl the hydrolyzed sample was redissolved in 1.2 ml of 0.2 M sodium citrate buffer (pH 2.2) and 1.0 ml was used for amino acid analysis while two 50-μl portions were measured in a scintillation counter (Birshtein *et al.*, 1971a). Although the accuracy of determination of counts per minute per micromole by this procedure was not great, the sharp decrease in specific activity at that Edman degradation step where *S*-carboxymethylcysteine was removed was sufficient to allow monitoring of this residue. The radioactivity of butyl acetate extracts was also measured to confirm the presence or absence of *S*-carboxymethylcysteine-^{14}C in the residual peptide.

Nomenclature. Tryptic peptides obtained by digestion of CNBr fragments from guinea pig γ_2 chain are numbered according to their position relative to the carboxyl-terminal end of the whole chain. Peptides derived from further enzymic splits of tryptic peptides retain the tryptic peptide number in their designation, *e.g.*, Pa-T32-3, but otherwise their number does not refer to a relative position in the molecule. Peptides obtained by the sequential use of three or more enzymes carry only the designation of the last enzyme used, *e.g.*, Pr2. However, for a given enzyme, the number in the names of these peptides is never repeated throughout the entire heavy chain. The enzymes used are signified as follows:

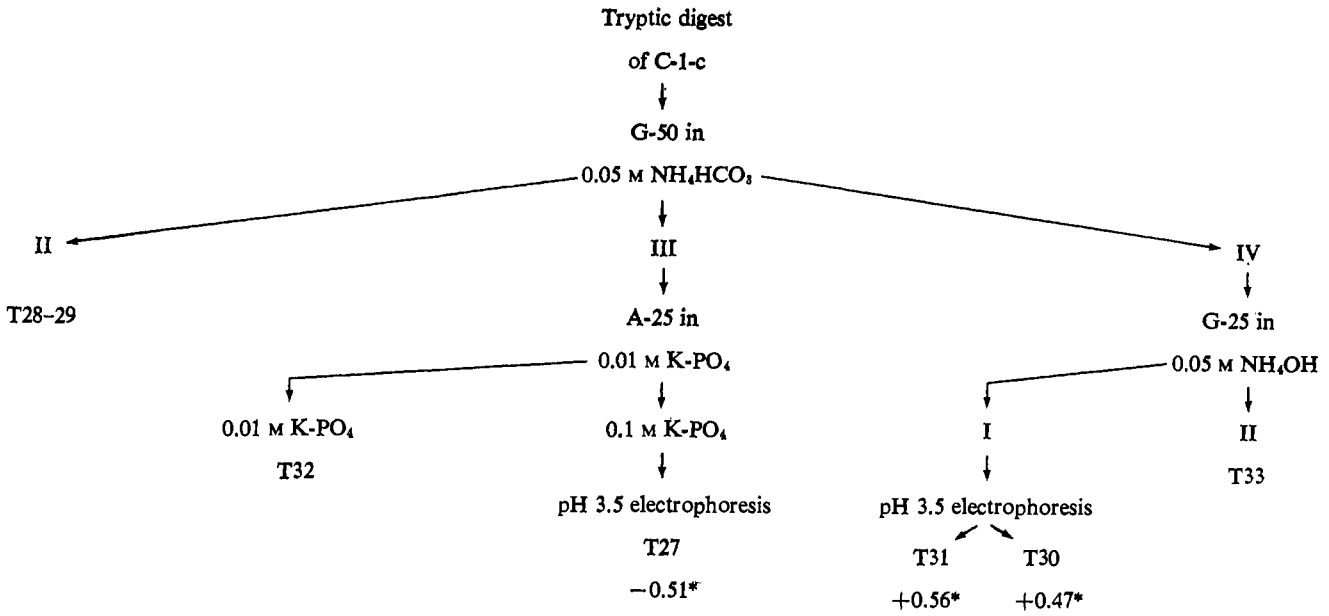

FIGURE 2: Flow diagram for the isolation of tryptic peptides from a digest of C-1-c. The procedures are described in the text. The A-25 signifies a column of DEAE-Sephadex, A-25. Electrophoretic mobilities (*) are given relative to lysine = +1.0, aspartic acid = −1.0, neutral amino acid = 0.

T, trypsin; Ch, chymotrypsin; Th, thermolysin; Pa, papain; Pe, pepsin; and Pr, pronase.

Results

Sequence of the Fragment C-5. As depicted in Figure 4, the N-terminal sequence of C-5 was found to be His-Glx-Ala-Leu and hydrazinolysis showed the C-terminal residue to be glycine. The composition of the three component tryptic peptides of C-5 is given in Table I. Since only T1 contains glycine and only T3 contains histidine, their order must be N-T3-T2-T1-C. Peptides T1 and T2 were sequenced directly as shown in Figure 4. Peptide T3 was digested with chymotrypsin and the products were fractionated on Sephadex G-25 (Figure 5). The analyses of pools I (C-T3-1) and II (C-T3-2) are also given in Table I. Since the composition of C-T3-1 is the same as that of the known N-terminal sequence of C-5 and because it contains the only leucine and alanine residues in T3, this peptide must be the N-terminal section of T3, and C-T3-2, which contains a lysine residue, must therefore be the C-terminal section. Upon electrophoresis at pH 6.5, C-T3-1 migrated as an acidic peptide. Thus its sequence is His-Glu-Ala-Leu. Peptide C-T3-2 was sequenced as shown in Figure 4. After removal of five residues by the Edman procedure, the remaining dipeptide, Glx-Lys, was found to migrate as a basic peptide upon electrophoresis at both pH 6.5 and 3.6. Thus it was presumed to be Gln-Lys.

TABLE I: Amino Acid Composition of the Fragment C-5 and of Peptides Obtained from It.[a]

	C-5	T1	T2	T3	C-T3-1	C-T3-2	Th1
Lys	0.99			1.0		1.0	
His	2.5			2.3	1.1	1.2	2.0
Arg	1.1		1.1				
Asp	1.1			1.0		1.0	1.1
Thr	1.1			0.85		1.2	
Ser	2.0	1.0	1.0				
Glu	2.1			1.9	1.1	1.2	
Pro	0.84	0.79					
Gly	1.2	1.1					
Ala	1.9		1.0	0.81	1.0		
Val	0.86			0.68		0.90	
Ile	0.99		0.96				
Leu	0.99			1.2	0.85		0.88

[a] Compositions are reported as moles of amino acid per mole of peptide.

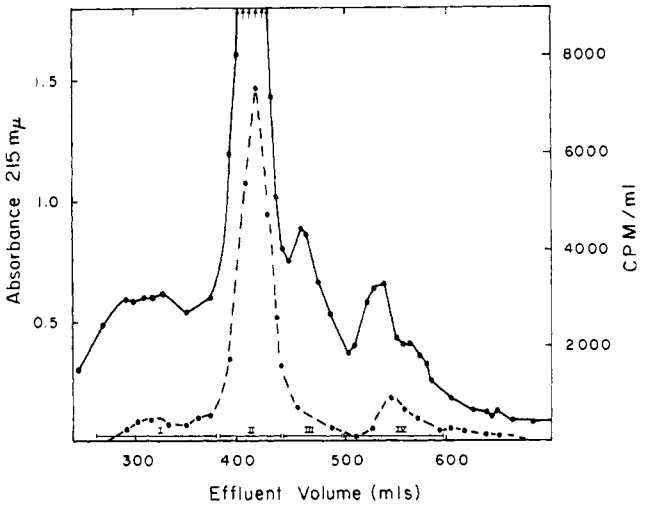

FIGURE 3: Fractionation of components in a tryptic digest of C-1-c. A digest of C-1-c (1.8 μmoles) was applied to a column of Sephadex G-50, fine (2 × 250 cm), equilibrated with 0.05 M NH_4OH. Elution was carried out with the same solution (5.7-ml fractions) and 0.1 ml of each fraction was taken for radioactivity measurements. Absorbance at 215 mμ (–·–) and counts per minute per milliliter (--●--).

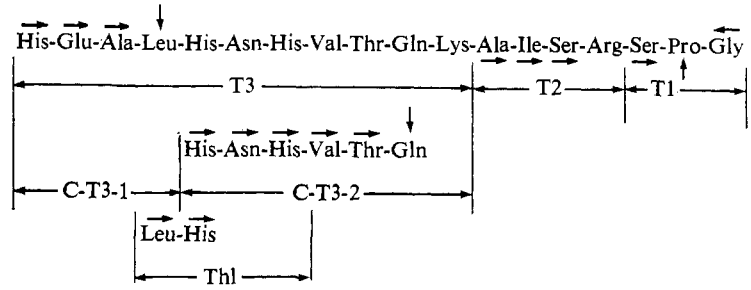

FIGURE 4: A schematic representation of the procedures and resulting data used to determine the sequence of C-5. Exact compositions of the peptides are given in Table I. →, removal of a residue by the Edman degradative procedure; ←, identification of residue by amino acid analysis after hydrazinolysis; ↑ or ↓, identification of N-terminal residue after dansylation. The sequence of residues in this fragment and its component peptides was determined by the Edman-dansyl procedure except for the residues in Th1, which were ordered using the subtractive Edman procedure.

TABLE II: Tryptic Peptides from C-1-c.[a]

	T27	T28	T29	T28–29	T30	T31	T32	T33
Lys		1.7	1.0	3.1		0.99	1.0	0.97
His							0.96	
Arg					0.90			
CMCys		0.60	1.8	2.5			0.70	
Asp	0.98	1.1	1.1	2.4		0.97	1.0	
Thr	1.0		1.9	2.2	0.91		1.9	1.0
Ser		1.1	0.78	1.4			1.9	2.1
Glu		1.2	1.2	2.1	1.2			1.1
Pro		7.1	3.8	9.3	1.1		1.2	0.93
Gly		2.0		2.0				
Ala				0.63			2.6	
Val		1.0		1.2	1.0	1.0	0.86	1.7
Ile		0.91		0.93	0.94			
Leu	1.0	1.1		1.2				
Phe		1.9		1.7				
Hsr	0.73							
Mobility[b]								
pH 3.6	−0.51	+0.10			+0.47	+0.56		
pH 6.5			−0.22		+0.05	+0.04		+0.39

[a] Compositions are reported as moles of amino acid per mole of peptide. [b] Mobility given relative to lysine = +1.0, aspartic acid = −1.0, neutral amino acid = 0.

The single Asx present in C-5 was defined by isolating peptide Th1 from a thermolysin digest of the fragment. Peptide Th1 was prepared from the whole digest by paper electrophoresis at pH 3.6 (relative mobility +0.91) and had the composition: (Leu,His,Asx,His) given in Table I. After two Edman degradative steps, the residual peptide, 1.0 Asx:0.8 His, moved as a basic peptide at pH 6.5 (relative mobility +0.69) indicating that the Asx residue was asparagine. The complete sequence of C-5 is, therefore, as given in Figure 4.

Tryptic Peptides from C-1-c and Their Alignment. The compositions of the tryptic peptides from C-1-c, isolated as described in Materials and Methods and schematized in Figure 2, are given in Table II. The amino acid analyses of T28 and T29, obtained from a tryptic digest of whole heavy chain, are also included in this table. The homoserine in T27 indicates that it is the C-terminal peptide. Fragment C-1-c was digested with chymotrypsin to get the overlapping peptides necessary to align the rest of the component tryptic peptides. The chymotryptic digest was fractionated on a column of Sephadex G-25 as depicted in Figure 6. When pool II (Figure 6) was chromatographed on a column of Dowex 1-X2 (Birshtein *et al.*, 1971b), a peptide fraction was eluted with 3% pyridine just after the breakthrough material and before the pyridine acetate gradient was applied to the column. The major component in this fraction was purified using paper electrophoresis at pH 3.6 and gave the analysis tabulated for Ch2 (Table III). The presence of homoserine indicates that Ch2 is the C-terminal chymotryptic peptide. It included T27 and, in addition (Lys_2,Pro_3,Ile,Phe). This additional section has the same composition as a peptide derived from T28–29 by either chymotryptic or peptic digestion, Ch- or Pe-T28–29-2, shown in Table IV. Thus the order of tryptic peptides at the C-terminal end of C-1-c is -T28/29–T27-C. The order of the peptides in this section was completed

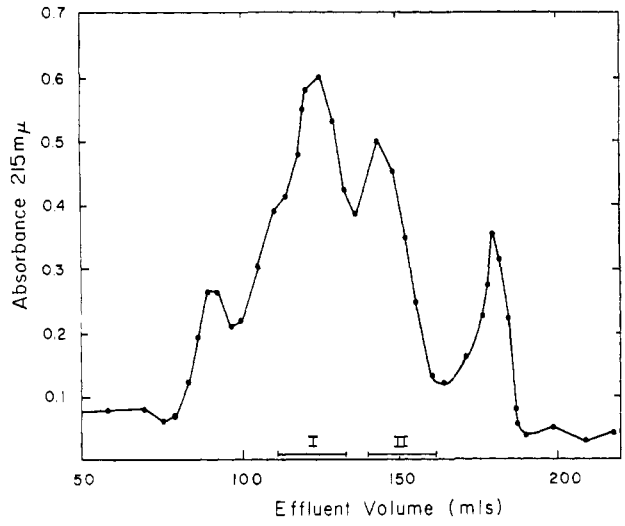

FIGURE 5: Separation of the components present in a chymotryptic digest of peptide T3. A digest of T3 (1.5 μmoles) was applied to a column of Sephadex G-25, fine (1.8 × 82 cm), in 0.05 M NH_4OH. Elution was with the same solution (3-ml fractions).

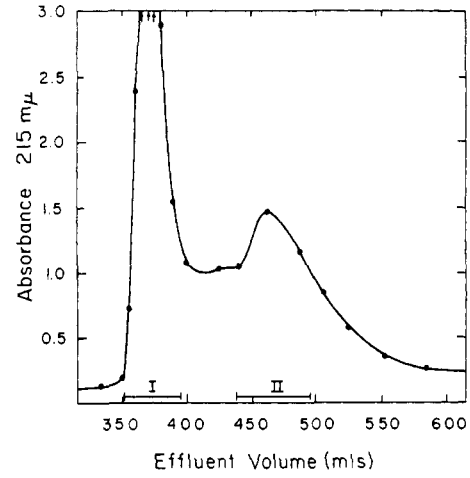

FIGURE 6: Fractionation of a chymotryptic digest of fraction C-1-c. The total digest of C-1-c (1.4 μmoles) was applied to a column of Sephadex G-25, fine (2 × 240 cm), equilibrated with 0.05 M NH_4OH. Elution was with the same solution (5-ml fractions).

by using N-terminal analysis. Both peptides T28–29 and T29 have N-terminal threonine residues while T28 lacks this residue entirely. In addition, peptide T28 contains the requisite two lysines to accommodate the C-terminal section of T28–29, Pe- or Ch-T28–29-2. Thus the order becomes T29-T28-T27-C.

The breakthrough material (pool I) from the chymotryptic digest of C-1-c (Figure 6) after recycling through the same column gave the analysis shown in Table III and proved to be a single peptide, Ch1. This peptide had the C-terminal sequence, Val-Phe, as determined using carboxypeptidase A for various times, and gave rise to T30 and T31, among other peptides, after tryptic digestion. Peptide Ch1 also contained all of the radiolabel from C-1-c. Like Ch1, the other peptide isolated from T28–29 by either chymotryptic or peptic digestion, Ch- or Pe- T28–29-1, shown in Table IV, contains all the radiolabel of T28–29 and has a C-terminal Val-Phe sequence. Thus Ch1 gives the alignment for tryptic peptides of -?-(T31,T30)T29-T28-T27-C.

In order to align T31 and T30 within this sequence, the lysine residues of peptide Ch1 were blocked by trifluoroacetylation to prevent tryptic cleavage at these positions and thus leave only the arginyl bond susceptible. The tryptic digest of this trifluoroacetylated derivative of Ch1 was fractionated on Sephadex G-25 and gave two components with the analyses listed as T-Ch1-1 and T-Ch1-2 in Table III. It is clear that T31 and T30 are found together in T-Ch1-2 along with a small fragment from another tryptic peptide. Since the arginine in T-Ch1-2 must be C-terminal and T30 is the only arginine-containing peptide, the alignment must be -T31-T30-T29-T28-T27-C.

Finally, the N-terminal residue of C-1-c, identified by the Stark procedure, was valine. The remaining peptides, T33 and T32, have N-terminal valine and alanine, respectively. Furthermore, the only other tryptic peptide from C-1-c with N-terminal valine is T31 and it has been placed within Ch1, which itself has an N-terminal serine. Thus the Ch1 peptide must begin with a small fragment of T32, and T33 must be the N-terminal tryptic peptide of C-1-c giving an order of N-T33-T32-T31-T30-T29-T28-T27-C (see Figure 7).

Sequence of the Fragment C-1-c. The sequences for the smaller tryptic peptides T27, T30, T31, and T33 were determined directly as indicated in Figure 7. Each of these four peptides has a single Asx or Glx residue, which was assigned on the basis of the electrophoretic mobility of the peptide. Since T27 migrates as an acidic peptide, even at pH 3.6, the Asx residue is aspartic acid (Table II). Both T30 and T31 behave as neutral peptides at pH 6.5 (Table II) and therefore must contain glutamyl and aspartyl residues, respectively. Finally, peptide T33 migrates as a basic peptide at pH 6.5 (Table II) so its Glx residue must be glutamine.

To sequence T28–29, a peptide of 31 residues containing 11 prolines and 3 *S*-carboxymethylcysteines, the isolation of a considerable number of component peptides from peptic, chymotryptic, papain, and pronase digests was necessary.

TABLE III: Peptides Used to Align Tryptic Peptides from C-1-c.[a]

	Ch1	CH2	T-Ch1-1	T-Ch1-2
Lys	2.1	2.3	0.97	1.4
Arg	0.80			0.96
CMCys	2.1		1.8	
Asp	3.3	1.0	2.3	1.0
Thr	2.9	1.1	1.8	1.2
Ser	2.2		1.8	1.5
Glu	3.4		2.3	1.3
Pro	6.4	3.1	5.1	1.0
Gly	2.1		2.0	
Ala	1.1			
Val	3.3		1.1	1.9
Ile	0.96	0.88		0.91
Leu	1.5	1.0	1.2	
Phe	0.92	0.77	0.77	
Hsr		0.95		
Mobility[a] pH 3.6		+0.54		

[a] Compositions and mobilities are expressed as indicated in Table II.

TABLE IV: Peptides Derived from T28–29 after Digestion with Various Enzymes.[a]

	Ch-T28–29-1	Pe-T28–29-1	Pe-T28–29-2	Ch-T28–29-2	Pr1	Pr2	Pr3	Pr4	Pr5	Pr6	Pa1	Pa2
Lys		0.84	2.0	2.2		1.0				1.1		
CMCys	0.71	2.1			0.47	0.51	0.85			0.94		
Asp	2.2	2.1			1.1		0.94		1.0			
Thr	1.9	2.3			0.91	0.89			1.0	0.92		
Ser	1.4	1.2						1.0	0.66		1.4	1.0
Glu	2.3	2.2			1.0		1.1		1.2			
Pro	6.0	7.4	3.1	3.0	3.2	1.1	3.0	1.0	1.6	1.5	0.98	0.95
Gly	2.2	1.7						2.0			1.9	1.2
Val	1.1	1.2									1.6	0.93
Ile			1.0	0.80								
Leu	1.0	0.75									0.88	
Phe	0.88	0.52	1.0	0.80								0.85
Mobility[a]												
pH 3.6					−0.27	+0.32	−0.56	+0.28	+0.18		+0.28	+0.18
pH 6.5	−0.12			+0.82								

[a] Compositions and mobilities are expressed as indicated in Table II.

The presence of radiolabeled carboxymethyl groups on all half-cystines in this section was used to confirm the presence of three easily reduced half-cystines and to place them in sequence. Both peptic and chymotryptic digestion of T28–29 yielded the same two peptides, Pe-T28–29-1 or Ch-T28–29-1 and Pe-T28–29-2 or Ch-T28–29-2 (Table IV). These two peptides were separated either by gel filtration on Sephadex G-25 (Pe-T28–29-1 eluted at 1.0 and Pe-T28–29-2 at 1.7 void volumes) or by paper electrophoresis at pH 6.5 (see Table IV). Peptide Pe- or Ch-T28–29-2, as indicated above, is the C-terminal section of T28–29 and T28. It was sequenced directly as depicted in Figure 7.

Table V shows that Pe-T28–29-1 contains all of the radiolabel originally present in T28–29. Separate portions of Pe- or Ch-T28–29-1 were digested with papain or pronase. The peptides in these digests were separated by gel filtration through Sephadex G-25 (2 × 240 cm) followed by high-voltage electrophoresis at pH 3.6 (see Table IV). Peptides Pr1, Pr2, Pr3, and Pr6 were eluted at the void volume of the Sephadex column while peptides Pr4, Pr5, Pa1, and Pa2 were eluted at about 1.2–1.4 void volume. Peptide Pa2 contains both the single valine and phenylalanine residues of Pe- or Ch-T28–29-1. Since the C-terminus of Pe or Ch-T28–29-1 is Val–Phe, Pa2 is the C-terminal section of the parent peptide. While peptide Pa2 contains one of the two glycine residues of T28, Pa1 contains both of them plus a leucine absent from Pa2. Thus Pa1 extends the sequence of Ch-T28–29-1 N terminal to Pa2 by (Leu,Gly). The exact sequence of this region of T28–29 was established by isolation and stepwise degradation (Figure 7) of the pronase peptide Pr4, which also contains two glycines.

TABLE V: Specific Activity of T28–29 and Its Component Peptides.[a]

	Prepn 1	Prepn 2	After No. of Degradative Steps 1	2	3	4	5
T28–29	257	–					
Pe-T28–29-1	235	113[b]					
Pr-3	61	40	11[c]	–	–	–	–
Pr-2	–[e]	44	59[d]	8	–	–	–
Pr-1	–	36	45[d]	30	39	30	30
Pr-6	–	98	46	46	–	–	–

[a] Expressed as (cpm/μmole) × 10^{-3}. [b] Labeled Pe-T28–29-1 from preparation 1 diluted with cold carrier peptide to give preparation 2. [c] Pr-3 from preparation 1 subjected to degradation. [d] Pr-2 and Pr-1 from preparation 2 subjected to degradation. [e] The dash indicates that specific activity was not determined.

The balance of residues necessary to account for T28 (Tables II and IV) was contained in the proline-rich peptide, Pr3. It was sequenced directly as indicated in Figure 7. Table V shows that Pr3 contains about one-third of the specific radioactivity of both T28–29 and its derivative, Ch-T28–29-1. After one step of the degradative Edman procedure, Pr3 lost most of its radiolabel (Table V). Thus radioactivity measurements confirmed that the N-terminal residue of Pr3 is *S*-carboxymethylcysteine. After four degradative steps, the remaining peptide from Pr3 (Glx,Asx), moved toward the anode at pH 6.5, while after the fifth degradative step, the residue Asx behaved as a neutral component on electrophoresis. Thus the last two residues of Pr3 appear to be Glu-Asn. The complete sequence of T28 is shown in Figure 7.

The sequence of the other section of T28–29, that of T29, was determined from degradation of two pronase peptides, Pr1 and Pr2, and was supported by the analyses of a third, Pr5, at stages of its degradation. The C-terminal peptide of T29, the lysyl peptide Pr2, had about one-third of the specific activity of the parent T28–29 and the derivative, Ch-T28–29-1. After the second step of the Edman degradation, most of

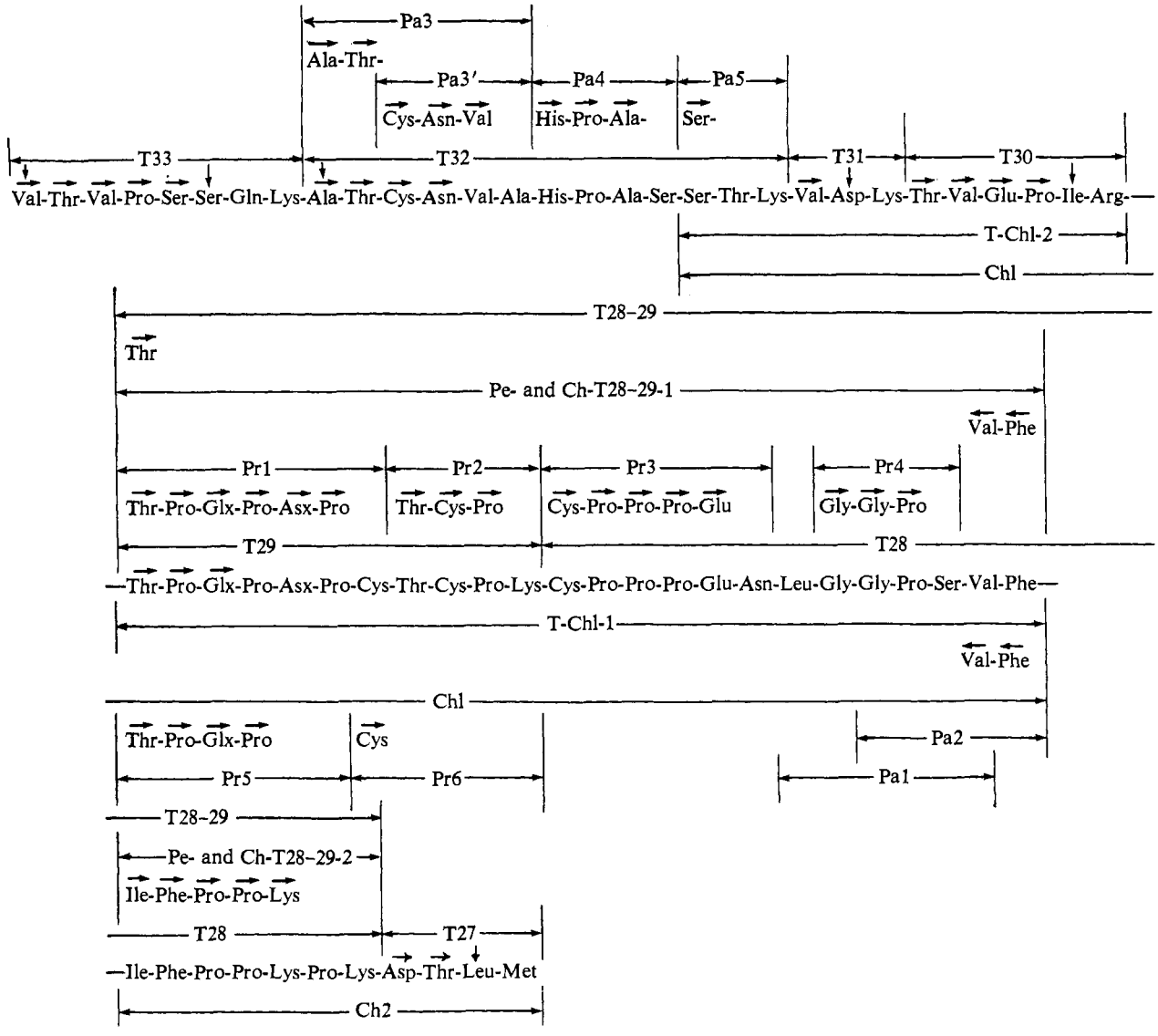

FIGURE 7: A schematic representation of the procedures and resulting data used to determine the sequence of C-1-c. Compositions of the peptides depicted are given in Tables II, III, IV, and VI. →, removal of a residue by the Edman degradative procedure; ←, identification of residue by amino acid analysis after its removal by the action of carboxypeptidase A; ↓, identification of N-terminal residue after dansylation. The sequence of residues in tryptic peptides T27, T30, T31, and T33 was determined by the Edman-dansyl procedure. The sequences of the larger peptides T28, T29, and T32 were obtained by using the subtractive Edman procedure on the tryptic peptides and their component parts as shown.

the radiolabel was lost (Table V). The sequence of this peptide was determined as schematized in Figure 7. The N-terminal sequence of T29 was obtained by sequencing Pr1, which like its parent peptide begins with the residues Thr-Pro-Glx (Figure 7). Table V indicates that Pr1 contained the remaining one-third of the label originally present in T28–29 and Ch-T28–29-1. This peptide retained its specific label through five cycles of the Edman procedure. An analysis of the residue after the sixth degradative step, both before and after acid hydrolysis, showed the presence of only *S*-carboxymethylcysteine. Figure 7 records a sequence of T28–29 in accord with the data so far discussed. The overall analyses of T28–29, T29, Ch-T28–29-1, and Pe-T28–29-1 indicate the presence of a partial residue of serine in the section covered by T29. The pronase peptide, Pr5, which appears to contain Asx and Ser at its C terminal end, was recovered in about 35% of the yield of Pr3. Thus it is possible that a Pro–Ser interchange may occur at position N-6 in the sequence of T29. The piece complementary to Pr5 is probably Pr6, and together these could make up an alternate T29. The peptide Pr6 contains two *S*-carboxymethylcysteines, one at the N-terminal position (Table V). Final evidence for a variant of T29 must await further characterization of this section of C-1-c. Unequivocal designation of Glx and Asx at position N-3 and N-5 of the T29 sequence was not possible using electrophoretic criterion.

The remaining tryptic peptide from C-1-c, T32, was sequenced as shown in Figure 7 using component papain peptides. The N-terminal sequence of T32 is Ala-Thr-Cys-Asx. Two peptides, Pa3 and Pa3′, each contain an *S*-carboxymethylcysteine residue. Peptide Pa3 has the same N-terminal sequence, Ala-Thr, as T32 and thus comes from the N-terminal end of the tryptic peptide. Peptide Pa3′ was derived from T32 after the source peptide had undergone two degradative steps. After removal of *S*-carboxymethylcysteine from Pa3′, the residual peptide (Asx,Val,Ala) behaved as a neutral

Rabbit	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Ile	Ser	Arg	Ser	Pro	Gly
Guinea pig	His	Glu	Ala	Leu	His	Asn	His	Val	Thr	Gln	Lys	Ala	Ile	Ser	Arg	Ser	Pro	Gly
Human (γ_1 and γ_2)	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly
Horse	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Val	Ser	Lys	Ser	Pro	Gly
Cow	His	Glx	Ala	Leu	His	Asx	His	Tyr	Met	Gln	Lys	Ser	Thr	Ser	Lys	Ser	Ala	Gly

FIGURE 8: A comparison of the amino acid sequences of the C-terminal fragment (C-5) from IgG of several species. The sequences of the octadecapeptides are taken from Givol and Porter (1965) for rabbit, Press *et al.* (1966) for human γ_1, Prahl (1967) for human γ_2, Weir *et al.* (1966) for horse, and Milstein and Feinstein (1968) for bovine C-5. To point out the regions of identity among the sequences, the rabbit C-5 was used as a standard and all residues in the other sequences differing from those of the rabbit at corresponding positions were excluded from the lined rectangle.

component during electrophoresis at pH 6.5. Thus the Asx residue appears to be asparagine. The balance of T32 can be accounted for by the compositions of Pa4 and Pa5 (Table VI). Since Pa5 contains the sole lysine in T32 it must be at the C-terminal end. Thus the papain peptides of T32 are ordered and sequenced as depicted in Figure 7.

Discussion

The amino acid sequence of the C-terminal octadecapeptide of γ chain from rabbit IgG (Givol and Porter, 1965) is now generally accepted as a standard for assigning immunoglobulins from other species to the IgG class. As Figure 8 clearly illustrates, the sequence of this section of heavy chain has been extensively conserved among mammalian species. The identity of a few residues at certain positions is so far unique for a given species, *e.g.*, Ala and Val at positions C-7 and C-11, respectively, for the guinea pig, Leu at positions C-4 and C-6 for the human, Lys and Val at positions C-4 and C-6 for the horse, and Ala, Lys, Thr, and Met at positions C-2, C-4, C-6, and C-10, respectively, for the cow. The residues Arg and Ile at positions C-4 and C-6, respectively, are thus far present only in the guinea pig and rabbit peptide. The overall sequence of the octadecapeptide has been found so characteristic of γ chain that an Ig previously considered a homolog of IgA from the horse (Rockey *et al.*, 1964) has been reclassified as IgG(T) on the basis of sequence data for the octadecapeptide (Weir *et al.*, 1966). The subclasses of human IgG and different allotypes within these subclasses all have C-terminal octadecapeptides clearly homologous with the prototype sequence from rabbit γ chain (Prahl, 1967). So far the only available sequence data for a human α chain is for the C-terminal peptide from a myeloma protein: Met-(Leu,Asx,Glx)-Ala-Gly-Thr-Cys-Tyr (Abel and Grey, 1969). This sequence would set IgA apart from IgG but still not differentiate it conclusively from IgM (Putnam, 1969).

The 65 residue fragment, C-1-c, contains three half-cystines which can be reduced under mild conditions and marked with carboxymethyl-^{14}C groups. This section can be formally aligned, by means of tryptic peptides including methionine residues, in a sequence of CNBr fragments as follows: N-(C-1-c)-(C-3)-(C-4)-(C-5)-C (D. C. Benjamin and Q. Z. Hussain, unpublished data). These three labeled half-cystines participate in forming inter-heavy-chain disulfide bonds (Oliveira and Lamm, 1971).

One section of C-1-c, peptide T28–29, contains the 3 labile half-cystines and 11 prolines within 31 residues. Hill and coworkers first reported the composition of a smaller, homologous peptide derived from the middle of rabbit heavy chain which had seven prolines in 21 residues (Hill *et al.*, 1966). This peptide was sequenced (Hill *et al.*, 1967; Cebra *et al.*, 1968). Hill's suggestion that the proline residues in this short peptide could lead to "open" or irregular structure where it occurred in the whole molecule was supported by placing adjacent to or within this region those peptide bonds most susceptible to papain and peptic cleavage (Givol and De Lorenzo, 1968) and by the electron microscopic visualization of this "hinge region" as a flexible position in the whole IgG molecule (Green, 1969). Thus, the extensive conservation among mammals of this proline-rich region, as depicted in Figure 9, may indicate its great importance for permitting conformational changes necessary for the effective functioning of antibody. A similar conservation of sequence in this section has been described for all four subclasses of human IgG (Frangione *et al.*, 1969).

On the other hand, Frangione and his colleagues have also pointed out the surprising variability of amino acid sequences among human IgG subclasses in the section of about ten residues containing most of the labile half-cystines and immediately N terminal to the hinge region (Frangione *et al.*, 1969). A comparison (Figure 9) of the major sequence of the section preceding the hinge from normal rabbit (Cebra *et al.*, 1968) and guinea pig IgG and from the major subclass of human IgG (Steiner and Porter, 1967), IgG(1), shows that variability among species in this section of the molecule is much greater than that found for any other "constant" section. Since antibodies of the IgG class can show remarkable

TABLE VI: Papain Peptides from T32.[a]

	Pa3	Pa3′	Pa4	Pa5
Lys				1.0
His			0.97	
CMCys	0.88	1.0		
Asp	1.0	0.99		
Thr	1.0			0.98
Ser			1.0	0.98
Pro			1.1	
Ala	1.9	1.0	1.0	
Val	1.1	0.91		
Mobility[a]				
pH 3.6	+0.06	−0.39	+0.66	
pH 6.5				+0.69

[a] Compositions and mobilities are expressed as indicated in Table II.

Guinea pig	Thr Lys Val Asp Lys Thr Val Glu Pro	Ile Arg Thr Pro Glx Pro Asx Pro Cys	Thr Cys Pro Lys Cys-
Rabbit	Thr Lys Val Asp Lys Thr Val Ala Pro	Ser Thr Cys	Ser Lys Pro Thr Cys-
Human (γ1)	Thr Lys Val Asp Lys Lys Val Glu Pro	Lys Ser Cys Asp Lys Thr His	Thr Cys Pro Pro Cys-

Guinea pig	-Pro Pro Pro Glu Asn Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
Rabbit	-Pro Pro Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
Human (γ1)	-Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met

FIGURE 9: A comparison of the amino acid sequences around the hinge region of γ chain from IgG of several species. The sequences shown are taken from Cebra *et al.* (1968) for rabbit IgG and from Steiner and Porter (1967) for human IgG(1). Three sequences are compared and when any two or three residues are identical at a given position they are included in the closed rectangles. No attempt was made to delineate homologies in the section containing a variable number of residues among the species, and this was left outside the closed rectangles.

differences in such secondary biologic activities (Bloch *et al.*, 1963) as activation of the complement system and initiation of anaphylactic reactions, a careful attempt to relate amino acid sequence and also pairing of half-cystines in the section just before the hinge with function might be fruitful. The finding that reduction of only two to three disulfide bonds, including the H–H interchain bond, would markedly decrease the ability of rabbit IgG to "fix" complement seems pertinent in this respect (Schur and Christian, 1964).

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