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Amino Acid Sequence of the V_H Region of a Human Myeloma Immunoglobulin (IgG New)†

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ABSTRACT: The amino acid sequence of the heavy-chain variable region of the human immunoglobulin. New has been determined. Since the amino terminus of the heavy chain was blocked, the sequence of residues 1-69 was established by digesting the appropriate CNBr fragment separately with trypsin, chymotrypsin, and thermolysin and sequencing the resulting peptides. The region from residues 70 to 120 was present in another CNBr fragment which was submitted directly to automatic Edman degradation. The result of this experiment extended the sequence to residue 100. The primary structure of the remaining portion of the V_H region was determined by automatic Edman degradation of a lysine-blocked tryptic peptide derived from this region which included residues 98-214. The sequence of the V_H region of New corresponds most closely to V_H sequences of proteins in the V_H II subgroup. This primary structure makes it possible to construct a model from the high-resolution electron-density map of protein New.

The three-dimensional structure of the Fab′ fragment of the human immunoglobulin New (IgG New) has been determined to a nominal resolution of 2 Å (Poljak et al., 1974). The light (L, λ) chain of IgG New has been sequenced and reported (Chen and Poljak, 1974). The study of the amino acid sequence of the heavy (H) chain was undertaken to obtain the necessary information for a complete interpretation of the 2.0-Å Fourier map of Fab′ New.

The amino acid sequence of IgG immunoglobulin H chains is usually divided into four homology regions, V_H, C_H1, C_H2, and C_H3, consisting of approximately 110 to 115 amino acids (Gally and Edelman, 1972). With the exception of well-characterized allotypic variants, human γ chains show sequence identity in their C_H regions as well as partial identity and strong homology in their variable V_H regions (Capra and Kehoe, 1975).

Two approaches were used to obtain peptides from the V_H region of New. In one case, Fab′ fragments were cleaved with CNBr and separated by gel filtration. This gave a fragment comprising residues 1-69. A combination of enzymatic and chemical degradation provided the required information to specify its sequence. The region from 70 to 120 was obtained via CNBr cleavage of partially reduced, alkylated heavy chain followed by separation of the fragments using gel filtration. Automatic sequenator runs on a CRBr fragment (residues 70-249) and on a tryptic peptide derived from this fragment (residues 98-214) gave results which allowed us to formulate a sequence for the entire heavy-chain variable region of protein New. This paper represents the evidence for the proposed sequence, gives a comparison of this sequence with other V_HII sequences, and discusses the relationship of this sequence with the electron-density map of protein New.

Experimental Section

Materials. Human myeloma immunoglobulin New (IgG_1, Gm(1+ 3− 4− 5−), λ) was purified from serum which had been kept frozen at −20 °C. Diethylaminoethylcellulose (0.9 mequiv/g) and carboxymethylcellulose (0.8 mequiv/g) were purchased from Serva. Sephadex G-100 and G-25 were obtained from Pharmacia. Tos-PheCH_2Cl trypsin, chymotrypsin, carboxypeptidase A (treated with DEF), carboxypeptidase B, and pepsin were purchased from Worthington Biochemical Corp. Thermolysin was obtained from Calbiochem. Dithiothreitiol, ethylenimine, cyanogen bromide, iodoacetic acid, dansyl chloride, and sequencing reagents were obtained either from Pierce Chemical Co. or from Beckman and were used fresh without further purification. Polyamide thin-layer sheets were purchased from Gallard-Schlesinger. Dansyl amino acid standards were prepared as described by Gray (1967a).

Preparation of H Chain from Protein New. IgG New was purified by precipitation with sodium sulfate followed by chromatography on diethylaminoethylcellulose and carboxymethylcellulose as described before (Rossi and Nisonoff, 1968). Mildly reduced and alkylated H chain was prepared by reducing a 20 mg/mL solution of IgG New in 0.2 M Tris-HCl (pH 8.5)-0.005 M EDTA-0.02 M dithiothreitol for 2 h at

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[1]Abbreviations used for immunoglobulins, their chains, and fragments are as recommended in *Bull. W.H.O. 30*, 447 (1964). Other abbreviations used are: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; DFP, diisopropyl fluorophosphate; Na_2EDTA, disodium ethylenedinitrilotetraacetate; PhNCS, phenyl isothiocyanate; Pca, pyrrolidonecarboxylic acid; Tos-PheCH_2Cl, 1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

room temperature, followed by alkylation with iodoacetic acid which was added to the reaction mixture to give a final concentration of 0.06 M. Alkylation was continued for 20 min in the dark with rapid stirring and was followed by dialysis (2–4 h) against 1 M propionic acid also in the dark. Preparative separation of H and L chains was accomplished using 5 × 100 cm Sephadex G-100 columns equilibrated with 1 M propionic acid and fitted with upflow adaptors. The H- and L-chain fractions were pooled, lyophilized, and stored as dried powders. Totally reduced and alkylated H and L chains were prepared by reduction of the polypeptides (10 mg/mL) in a solution containing 8 M guanidine hydrochloride, 0.2 M Tris-HCl (pH 8.5), 0.005 M EDTA, and 0.1 M dithiothreitol for 2 h at 40 °C. After alkylation with iodoacetic acid (added to a final concentration of 0.3 M), the sample was dialyzed against freshly prepared 8 M urea–1 M acetic acid buffer and filtered through a 5 × 100 cm Sephadex G-100 column equilibrated with the same buffer. The H and L chains thus obtained were extensively dialyzed against distilled water, lyophilized, and stored as dry powders.

Preparation of Fab′ Fragments. Purified IgG New was digested with papain as described before (Rossi and Nisonoff, 1968) using a papain/IgG New ratio of 1:100. The digest was dialyzed against 0.01 M phosphate buffer, pH 8.0, and chromatographed on a diethylaminoethylcellulose column equilibrated with the same buffer. The eluted Fab′ fragment was concentrated by precipitation with 75% saturated ammonium sulfate, centrifuged, redissolved, extensively dialyzed against distilled water, and lyophilized.

CNBr Cleavage of Fab′ and H Chains: Separation of CNBr Fragments. Lyophilized Fab′ fragments or H chains were dissolved in 70% formic acid and CNBr was added to give final concentrations of 20 mg/mL protein, 60 mg/mL CNBr. Digestions were carried out for 18 h at room temperature, terminated by the addition of 5 volumes of ice-cold distilled water, and immediately lyophilized. The freeze-dried digest of Fab′ New was dissolved in 1 M acetic acid and separated into three fractions by gel filtration on Sephadex G-100 columns, as shown in Figure 1A. Peaks I, II, and III (Figure 1A) obtained after gel filtration were totally reduced and aminoethylated or were alternatively oxidized with performic acid, following the procedures of Raftery and Cole (1966) and Hirs (1967), respectively, and again chromatographed on a Sephadex G-100 column. After this treatment, peaks I and II gave an essentially identical pattern of the type shown in Figure 1B; the elution position of the minor peak III remained as it had been in the first chromatographic separation (Figure 1A). Similar optical-density profiles and separations were obtained using Sephadex G-100 columns equilibrated with 6 M guanidine hydrochloride, 0.02 M Tris-HCl, pH 8.0. The relative proportions of peaks I and II varied in the different runs. Since, after reduction and aminoethylation, peak I (Figure 1A) gave the same components as did peak II, peak I was considered to consist of aggregated material.

CNBr-cleaved H chains were fractionated by gel filtration in 1.0 M propionic acid on 2.5 × 100 columns of Sephadex G-75.

Reduction and Alkylation of the CNBr Fragments of Protein New Heavy Chain. The CNBr fragment (cut 1, Figure 2A) (200 mg) was dissolved in 6 M guanidine hydrochloride, containing 50 mM Tris and 5 mM EDTA, pH 8.5, and the solution was kept at 50 °C for 30 min. Dithiothreitol (40 mM) was added, the solution flushed with N_2, and the reduction allowed to proceed at 50 °C. The solution was cooled to 25 °C and 50 μCi of [^{3}H]iodoacetic acid was added and allowed to stand in the dark at 25 °C for 15 min. The solution was then dialyzed first against distilled water and then against 6 M urea containing 0.1 M glycine and 1.0 M propionic acid. Finally, the solution was passed through a column (4.5 × 100 cm) of Sephadex G-100 and eluted with 6 M urea containing 1.0 M propionic acid and 0.1 M glycine.

Radiosuccinylation of a CNBr Fragment (Fraction A, Figure 1B) from the Heavy Chain. The carboxymethylated CNBr fragment (fraction A, Figure 1B) (400 mg) was dissolved in 6 M guanidine hydrochloride and the pH of the solution was adjusted to 8.5 with NaOH. [^{14}C]Succinic anhydride (50 μCi) (New England Nuclear) was added and the pH of the reaction mixture kept at 8.5 with 0.1 M NaOH. The reaction was carried out for 15 min and then 100 mg of cold succinic anhydride was added and, after another 15 min at pH 8.5, the reaction mixture was dialyzed against distilled water, frozen, and lyophilized.

Enzymatic Digestion of CNBr Fragments from the Heavy Chain. Aminoethylated or performic acid oxidized H chain CNBr fragments were digested with Tos-PheCH_2Cl trypsin, chymotrypsin, or thermolysin for 4 h at room temperature in 0.1 M NH_4HCO_3 using an enzyme:substrate ratio of 1:100. Digestions with pepsin were carried out in 0.1 M formic acid under similar conditions. Digestion of purified peptides with chymotrypsin, trypsin, or pepsin was performed as proposed by Smyth (1967). A similar procedure was used for thermolysin digestions which were conducted at 45 °C. Digestions with carboxypeptidases A and B were performed as described by Ambler (1967).

Fractionation and Purification of Peptides. Peptides obtained by enzymatic digestion of H-chain fragments were separated by gel filtration on 2.5 × 100 cm columns of Sephadex G-25 (fine) equilibrated with 0.1 M NH_4OH. The column effluents were monitored by absorption readings at 280 and 232 nm, which permitted the digests to be divided into fractions which were then lyophilized, redissolved in pH 6.5 buffer (10% pyridine, 0.6% acetic acid, 89.6% water), and submitted to high-voltage electrophoresis at pH 6.5 using gradients of 50 V/cm for periods of time ranging from 60 to 400 min in Varsol-cooled tanks. Cysteic acid, glutamic acid, aspartic acid, threonine, leucine, histidine, arginine, and lysine were used as electrophoretic markers and run side by side with the peptides under investigation. After electrophoresis, guide strips were stained with ninhydrin (0.2% in acetone), with a chlorine stain (Reindel and Hoppe, 1954), and with stains specific for arginine and tryptophan (Bennett, 1967). Subsequent purification steps included paper chromatography and high-voltage electrophoresis at different pH values. The papers used in chromatography were previously washed by descending chromatography with distilled water containing 0.1% EDTA. The chromatographic solvent consisted of 1-butanol–acetic acid–pyridine–water (25:3:10:12). Further peptide purification was achieved by high-voltage electrophoresis using a pH 2.0 buffer (formic acid–acetic acid–water 25:87:888), a pH 4.7 buffer (acetic acid–pyridine–water 1:1:78), and a pH 3.5 buffer (acetic acid–pyridine–water 10:1:189). When a two-dimensional or a "three-dimensional" separation procedure was required to purify a given peptide, its location on paper was established using a weak ninhydrin stain (0.2% ninhydrin in acetone). As soon as the peptide became visible, the area containing it was cut out and washed with a 1:1 mixture of ethanol–acetone. Peptides were eluted from paper (using 6 M HCl) into 50- or 100-μL glass capillaries which were sealed and kept at 110 °C (18–24 h) prior to amino acid analysis. Alternatively, peptides were eluted with 0.1–0.3 mL of a 1:1

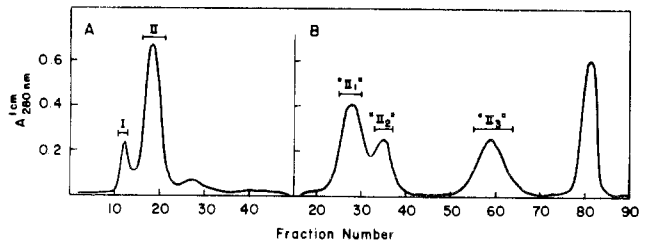

FIGURE 1: (A) Separation of fragments from CNBr-treated Fab' New on a 2 × 90 cm column of Sephadex G-100 in 1 M acetic acid using an upward-flow adaptor. Cuts were taken as indicated. (B) Gel filtration of cut 2 (Figure 1A) after reduction and aminoethylation or after performic acid oxidation. The separation was carried out exactly as described for Figure 1A and the cuts indicated were taken for further work.

mixture of water–pyridine and reserved for sequence determination.

Amino Acid Analyses. Quantitative amino acid analyses were performed by the method of Moore et al. (1968) using a Durrum D-500 or a Beckman 121 M amino acid analyzer.

Sequencing Techniques. Sequential degradation of purified peptides was performed using the Edman reaction (Edman, 1956). After each degradation step, aliquots of peptides were removed to determine the newly exposed NH_2-terminal residue, as proposed by Hartley (1970) and Gray (1967b). The dansyl derivatives of amino acids were identified by chromatography using the procedure of Woods and Wang (1967) on 4.5 × 7.5 cm polyamide sheets. Chromatography was performed using 1.5% formic acid in the first dimension, benzene–glacial acetic acid (9:1) in the second dimension, and ethyl acetate–methanol–acetic acid (20:1:1) in the third dimension. Automatic sequencing was performed by using a JEOL JAS-47K sequence analyzer. A standard protein program with a quadrol buffer system and a peptide program with dimethylallylamine buffer system was employed according to the procedure of Hermodson et al. (1973). The double-cleavage program was used during acid cleavage. The thiazolidine derivatives released were converted in 1 N HCl at 80 °C for 10 min to phenylthiohydantoin (Pth) derivatives which were identified by gas chromatography (Pisano et al., 1972) and by thin-layer chromatography (Summers et al., 1973). Finally, a portion of the Pth derivatives was hydrolyzed in 57% HI at 130 °C for 18 h and the resulting amino acids were determined by amino acid analysis (Smithies et al., 1971).

Results

Two approaches were used to obtain variable-region fragments of protein New heavy chains. In one approach, the Fab' portion of New was prepared and cleaved with CNBr, and the fragments were isolated by gel filtration. The heavy-chain region, from residue 1 to 69, was obtained in fairly good yield this way, but difficulties were encountered in isolating the region from residue 70 through the start of the constant region in pure form and in high enough yield. To circumvent this difficulty, a complementary strategy, which involved CNBr cleavage of isolated heavy chains, was employed. In this case, a fragment spanning residue 70 to 249 was obtained pure and in high yield, whereas the NH_2-terminal CNBr fragment was not pure enough for further use. Tryptic peptides were obtained, however, from this region and these peptides also served as a check on the validity of the results obtained for the sequence of residues 1–69 using the Fab' portion as mentioned above. To facilitate the presentation of the results, the isolation and characterization of the CNBr fragments, using each approach, will be discussed first. This will be followed by the sequence data on each part of the heavy chain which enabled us to propose a primary structure for the V_H region of protein New.

TABLE I: Amino Acid Composition of CNBr Fragments from Fab' and the Heavy Chain of Protein New.[a]

Amino acid	Peak[b] II_1	Peak[b] II_2	Peak[b] II_3	Res 1–69	Peak[c] A	Peak[c] B
Lys	9.7	6.8	0.1	0	7.1	0.4
His	4.2	2.3	1.1	1	2.1	1.2
NH_3						
Arg	4.7	14.6	4.9	5	14.2	5.0
Trp	Nd[f]	Nd[f]	2.0	2		2
Asp	15.2	12.5	4.4	4	13.2	4.1
Thr[d]	21.3	15.3	8.8	9	14.6	9.3
Ser[d]	30.3	22.5	7.2	8	21.6	8.5
Glu	22.9	10.1	7.5	7	11.0	8.4
Pro	17.1	10.8	5.1	5	11.3	6.3
Gly	17.1	12.6	7.4	7	13.4	8.5
Ala	21.5	13.8			12.9	
½-Cystine[e]	3.0		0.7	1		1.1
Val	19.1	16.1	6.3	6	17.8	7.1
Met[e]			0.6	1		0.7
Ile	5.5	4.1	1.2	1	4.6	1.3
Leu	17.6	14.1	6.4	6	15.0	7.2
Tyr	9.2	2.2	4.1	4	2.3	3.6
Phe	5.8	3.8	2.1	2	4.1	1.8
Total	227	168	69	69	165	76

[a] Values are reported as residues per mole based on assumptions about the size of the CNBr fragments. [b] Figure 1B. [c] Figure 2B. [d] Not corrected for partial destruction during hydrolysis. [e] Cysteine and methionine analyzed as aminoethylcysteine and homoserine lactone, respectively. Tryptophan was determined directly on the amino acid analyzer after hydrolysis with methanesulfonic acid. [f] Nd, not determined. Values are listed as residues/mol.

Isolation and Characterization of the CNBr Fragments from the Fab Portion of Protein New. The CNBr cleavage products of the Fab' portion were separated by gel filtration as shown in Figure 1A. The material represented by each peak was reduced, aminoethylated, and rerun on the same column. The pattern shown in Figure 1B was obtained with both peaks I and II from Figure 1A, suggesting that peak I was an aggregated form of peak II. A pattern identical to that shown in Figure 1B was also seen when peaks I and II were oxidized with performic acid prior to rechromatography. Based on amino acid composition (Table I) and NH_2-terminal data, the following assignments could be made: Peaks "I_1", "II_2", and "II_3" (Figure 1B) were: L chains, heavy-chain residues 70 to approximately 249, and heavy-chain residues 1 to 69, respectively. Peaks "II_1" and "II_2" were not entirely pure but peak "II_3" was homogenous and was, therefore, used as the principal starting material for sequence determination of the NH_2-terminal region (residues 1–69), which will be described in a subsequent section.

Isolation and Characterization of the CNBr Fragments from the Heavy Chain of Protein New. After cleavage of the heavy chain with CNBr and gel filtration on Sephadex G-75, an elution pattern (Figure 2A) was obtained. Based on size, amino acid composition, and NH_2-terminal data, the fractions were assigned as follows: Fraction 1, the region from residues 1 to 249 (residues 1–69 linked to 70–249 by a disulfide bridge); fraction 2, residues 250 to 428; and fraction 3, residues 426 to 443. Since fraction 1 contained the variable region, it was reduced, alkylated with [^{3}H]iodoacetic acid, and chromatographed on Sephadex G-100, as shown in Figure 2B. Peak A

TABLE II: Amino Acid Composition of the Tryptic Peptides of CNBr Fragment II_3 from Fab' New.[a]

Amino acid	T_0	T_1	T_2	T_3	T_4	T_5	T_6	Sum. T_1-T_6
AE-Cys[d]		1.04 (1)						1
His					1.1 (1)			1
Arg	0.9 (1)	0.9 (1)	1.0 (1)	0.9 (1)	0.9 (1)	1.0 (1)		5
Trp			1.0 (1)		1.0 (1)			2
Asp			2.1 (2)		2.0 (2)			4
Thr		2.1 (2)	2.6 (3)		2.8 (3)		1.0 (1)	9
Ser	0.8 (1)	3.1 (3)	2.6 (3)		1.0 (1)	1.0 (1)		8
Glu	3.9 (4)	5.2 (5)	0.2 (0)	1.0 (1)	1.1 (1)			7
Pro	0.8 (1)	1.9 (2)	0.2 (0)	2.0 (2)	1.1 (1)			5
Gly	2.1 (2)	2.2 (2)	1.2 (1)	1.0 (1)	3.0 (3)			7
Ala			0.1 (0)					
Val	2.0 (2)	1.9 (2)	1.9 (2)		1.0 (1)		1.0 (1)	6
Met							0.3[b] (1)	1
Ile			0.1 (0)		1.0 (1)			1
Leu	1.9 (2)	3.9 (4)	0.2 (0)		1.8 (2)			6
Tyr			2.0 (2)		2.1 (2)			4
Phe			1.0 (1)		1.0 (1)			2
Total	13	22	16	5	21	2	3	69
% Yield[c]	26	30	15	33	15	27	23	
Res no.	1-13	1-22	23-38	39-43	44-64	65-66	67-69	1-69

[a] Values listed as residues/mol. Assumed integral values are given in parentheses. [b] Homoserine lactone. [c] Estimated from amount of material digested. [d] AE-Cys, aminoethylcysteine.

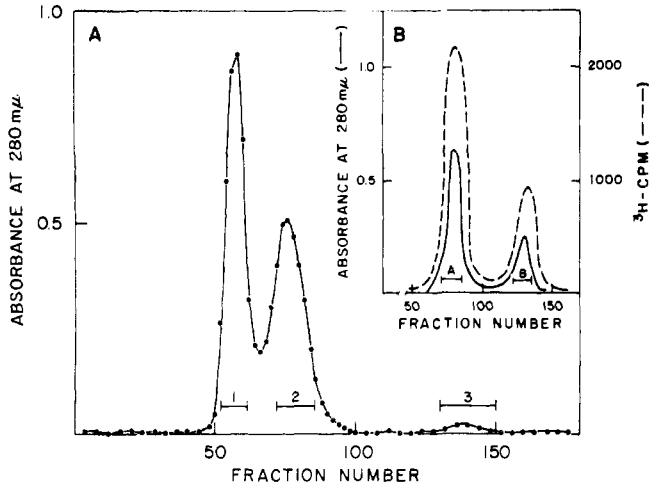

FIGURE 2: (A) Gel filtration of CNBr-cleaved heavy chain on a 2.5 × 100 cm column of Sephadex G-75 equilibrated with 1 M propionic acid. Five-milliliter fractions were collected and pooled as indicated. Pool 1 was reduced and alkylated with [^{3}H]iodoacetic acid (see text). (B) Reduced carboxymethylated pool 1 (Figure 2A) run on a 4.2 × 100 cm column of Sephadex G-100 equilibrated with a solution containing 6 M urea, 0.1 M glycine, and 1.0 M propionic acid. Both absorbance at 280 nm and radioactivity were used to detect the material. Fractions of 10 mL were taken and pooled as indicated.

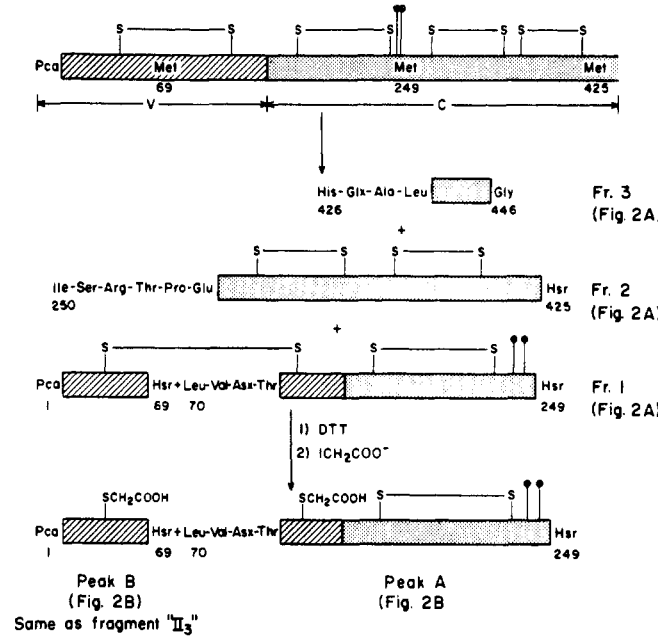

FIGURE 3: Scheme for the CNBr cleavage of protein New heavy chains and the placement of the CNBr fragments. The location of the disulfide bridges are shown as well as the correlation of the fragments with the elution pattern shown in Figure 2A,B; the two vertical lines next to the second disulfide bridge represent alkylated SH groups obtained by mild reduction and alkylation of IgG New.

(Figure 2B) covered the region from residue 70 to 249, while peak B contained the region which comprised residues 1 to 69. The amino acid analysis of the material in peaks A and B (Figure 2B) is given in Table I. A schematic diagram of the assignment of CNBr fragments in the heavy chain is presented in Figure 3.

A peptide was isolated from a tryptic digest of cut 2 (residues 250-428) (Figure 2A) which had the partial sequence: Asp-(Glu, Leu, Thr)-Lys. This corresponds to positions 356-360 in the constant region of IgG Gm(1) (a+) immunoglobulins (Thorpe and Deutsch, 1966) and confirms the assignment of the Gm(1) (a+) allotype to protein New.

Separation and Sequence Determination of the Tryptic Peptides from the CNBr Fragment II_3 (Residues 1-69). After digestion of the CNBr fragment II_3, with trypsin, the peptides were separated first by gel filtration, then by paper electrophoresis at pH 6.5, and finally by paper chromatography or by paper electrophoresis at pH 4.7. The amino acid composition of the isolated tryptic peptides are given in Table II. A summary of the sequence determination of this region is shown

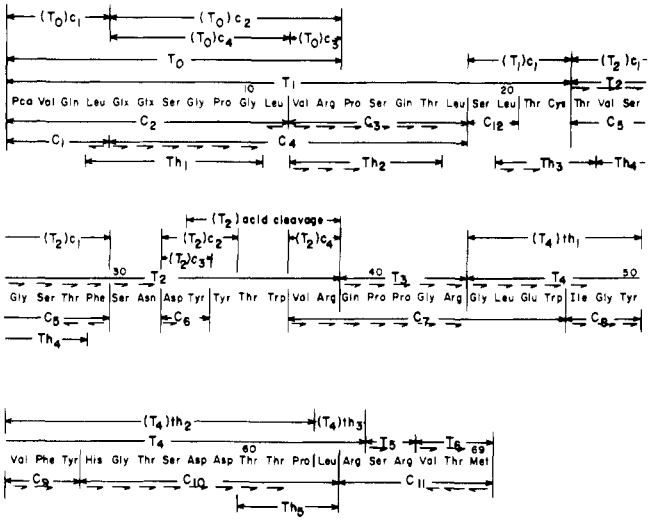

FIGURE 4: Summary of the evidence for the proposed sequence of CNBr fragment II_3 (Figure 1B). Tryptic, chymotryptic, and thermolytic peptides are indicated by T, C, and Th, respectively. Chymotryptic and thermolytic peptides derived from tryptic peptides are indicated as $(T_n)c_i$ or $(T_n)th_i$, respectively. (→) Residue identified after dansyl or subtractive Edman degradation. (←) Residue identified after carboxypeptidase digestion.

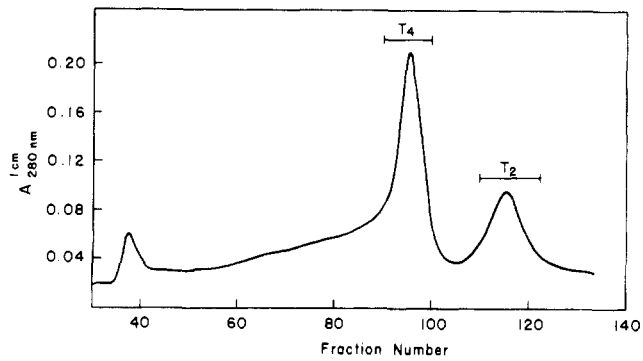

FIGURE 5: Separation of tryptic peptides T_2 and T_4 by gel filtration on a 2.5 × 90 cm column of Sephadex G-50 superfine equilibrated with 1 M acetic acid. Fraction size was 5 mL.

in Figure 4 and a description of the evidence for each tryptic peptide is presented below.

T_0. This peptide was detected after paper electrophoresis at pH 6.5 by the chlorine reagent of Reindel and Hoppe (1954). Since it was ninhydrin negative, we concluded that it had a blocked NH_2 terminus (presumably because of a pyrollidonecarboxylic acid (Pca residue). Satisfactory yields were obtained only after overnight digestion with trypsin. This was due to the slow rate of hydrolysis of an Arg–Pro bond. For determination of the internal sequence of T_0, chymotryptic digestion was employed. Although four peptides were obtained as shown in Figure 4, it was possible to deduce their arrangement, since $(T_0)c_4$ and $(T_0)c_3$ were both part of $(T_0)c_2$. Because $(T_0)c_1$ has a blocked NH_2 terminus, they were placed as shown in Figure 4. Assuming that $(T_0)c_1$ had a NH_2-terminal (PCA) group, its sequence was established by the ordered release of leucine followed by glutamine with carboxypeptidase A (CPA). $(T_0)c_3$ was shown to be Val-Arg by dansylation. $(T_0)c_4$ was subjected to six steps of dansyl Edman degradation and CPA digestion with the results shown in Figure 4. Due to the lack of adequate material it was not possible to assign the amides to the 2 Glx residues (positions 5 and 6), although it was clear from the electrophoretic mobility at pH 6.5 that one of the 2 Glx residues must be glutamine.

T_1. This peptide was ninhydrin negative and contained aminoethylcysteine, which means that it probably included either residue 23 or 77 based on homology with the sequence of other V_H regions. Chymotryptic digestion of T_1 gave $(T_1)c_1$ which must have been at the COOH terminal, since it contained aminoethylcysteine. The other part of T_1 was not recovered. The sequence of $(T_1)c_1$ was established by the dansyl Edman degradation and the sequence of the NH_2-terminal section of T_1 was obtained by degradation of C_3, an overlap peptide isolated from a chymotryptic digest of the CNBr fragment II_3. The results are shown in Figure 4 but will be discussed in a subsequent section.

T_2. This peptide has very low solubility in the aqueous solvents used for gel filtration and paper electrophoresis. It also tended to associate with itself and with peptide T_4. The similarity in size and amino acid composition hampered the efficient separation of these two peptides but almost complete resolution was obtained using Sephadex G-50 superfine, as shown in Figure 5. The NH_2-terminal sequence analysis of T_2 was carried out for nine steps by automatic Edman degradation and confirmed for seven residues by the isolation and sequencing of $(T_2)c_1$. To complete the structure of T_2, it was digested with chymotrypsin and the peptides were separated by paper electrophoresis and paper chromatography. Four peptides were isolated in high enough yields to ensure that they were derived from T_2 and not from a contaminating peptide. The sum of the four peptides did not account for all the amino acids in T_2, so another method of cleavage (mild acid hydrolysis) was employed. This yielded a hexapeptide which was easily isolated in pure form, since it was markedly retarded on columns of Sephadex G-25. This peptide, shown in Figure 4, together with the seven residues at the NH_2 terminus of T_2 accounted for all amino acids in T_2 except for one aspartic acid. This aspartic acid was placed unequivocally at the eighth position in T_2, since it was the NH_2-terminal residue of $(T_2)c_2$ and $(T_2)c_3$, peptides which overlapped the acid-cleavage peptide derived from T_2. Although dansyl-Edman degradation of the acid-cleavage peptide placed threonine before tryptophan, the results were not as definitive as we would have liked. For this reason (and others), we decided to compare the proposed sequence in this region with the Fourier map of electron density of Fab′ New at 2-Å resolution (Poljak et al., 1974). In this map, the aromatic side chains of Phe-29, Tyr-33, Tyr-34, and Trp-36 can be unequivocally positioned providing a precise stereochemical framework for the location and identification of the remaining side chains. It is also worth mentioning here that lack of correlation with the Fourier map allowed us to rule out provisional sequences obtained earlier on incompletely purified T_2 material.

T_3. This peptide was isolated in high yield after paper electrophoresis at pH 6.5 and paper chromatography. Because its mobility at pH 6.5 indicated that it had a net positive charge, the NH_2-terminal Glx residue must be glutamine. The sequence was determined in a straightforward manner by the dansyl Edman procedure and confirmed by subtractive Edman degradation.

T_4. As with T_2, this peptide was insoluble after tryptic digestion. In contrast to T_2, however, it was solubilized below pH 4 and was purified by chromatography on Dowex 50-X4. Later it was also isolated during the purification of T_2 by gel filtration, as shown in Figure 5. The amino acid composition of T_4 isolated by both methods was in agreement and is given in Table II. The sequence of T_4 was elaborated by a series of combined approaches. Subtractive Edman degradation pro-

TABLE III: Amino Acid Composition of the Chymotryptic Peptides from the CNBr Fragment III_3.[a]

Amino acid	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
His										1.0 (1)	
Arg			1.1 (1)	1.0 (1)			2.1 (2)				2.1 (2)
Trp							0.9 (1)				
Asp						1.0 (1)				2.1 (2)	
Thr			1.0 (1)	0.9 (1)	2.0 (2)					3.1 (3)	1.0 (1)
Ser		0.9 (1)	1.0 (1)	1.9 (2)	2.0 (2)					1.0 (1)	1.0 (1)
Glu	2.0 (2)	4.1 (4)	1.1 (1)	3.3 (3)			2.0 (2)				
Pro		0.9 (1)	1.1 (1)	2.0 (2)			2.0 (2)			1.1 (1)	
Gly		2.1 (2)		1.9 (2)	1.1 (1)		2.1 (2)	0.9 (1)		1.1 (1)	
Val	1.0 (1)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)		0.9 (1)		1.0 (1)		1.0 (1)
Met											0.4[b] (1)
Ile								1.1 (1)			
Leu	1.1 (1)	1.9 (2)	1.0 (1)	1.9 (2)			1.0 (1)			1.0 (1)	
Tyr						1.0 (1)		1.1 (1)	0.9 (1)		
Phe					1.1 (1)				1.1 (1)		
Total	4	11	7	14	7	2	11	3	3	10	6
% Yield[c]	27	21	18	23	19	15	17	30	21	27	29
Res no.	1-4	1-11	12-18	5-18	23-24	32-33	37-47	48-50	51-53	54-63	64-69

[a] Values listed as residues/mol. Assumed integral values are given in parentheses. [b] Homoserine lactone. [c] Estimated from the amount of material digested.

TABLE IV: Amino Acid Composition of Thermolytic Peptides From CNBr Fragment II_3.[a]

Amino acid	Th_1	Th_2	Th_3	Th_4	Th_5
AE-Cys[b]			1.0 (1)		
Arg		0.8 (1)			
Thr		1.0 (1)	2.0 (2)	1.1 (1)	2.1 (2)
Ser	0.9 (1)	1.0 (1)		2.0 (2)	
Glu	2.0 (2)	1.1 (1)			
Pro	0.9 (1)	0.9 (1)			0.0 (1)
Gly	2.1 (2)			1.0 (1)	
Val		1.9 (1)		1.0 (1)	
Ile	1.0 (1)				
Leu			0.9 (1)		0.9 (1)
Total	7	6	4	5	4
% Yield[c]	33	28	27	23	23
Res no.	4-10	12-17	20-23	24-28	60-63

[a] Values are listed as residues/mol. [b] Aminoethylcysteine. [c] Estimated from the amount of material digested.

vided information for the first five residues, although the tryptophan residue at position 4 was definitively assigned only after isolation and sequencing of C_7, a peptide derived from chymotryptic digestion of CNBr fragment II_3. When T_4 was digested with thermolysin, three peptides were obtained which accounted for all of T_4. $(T_4)th_1$ comprised the NH_2-terminal seven residues of T_4, five of which had already been positioned. The remaining two residues were fixed by CPA digestion of $(T_4)th_1$ which released tyrosine and independently by the sequencing of C_8. The middle region of T_4 was contained in peptide $(T_4)th_2$ whose sequence was established by dansyl Edman degradation and confirmed by sequencing the chymotryptic peptides C_9 and C_{10} (derived from CNBr fragment II_3). The COOH-terminal dipeptide needed to complete the sequence of T_4 was $(T_4)th_3$ which turned out to be Leu-Arg.

T_5. This dipeptide, Ser-Arg was isolated by paper electrophoresis and paper chromatography.

T_6. This was a tripeptide, Val-Thr-Hsr, isolated in the same way as T_5. Subtractive Edman degradation was used to establish its sequence. Since it was the only tryptic peptide that contained homoserine, it must have come from the COOH terminus of CNBr fragment II_3.

Ordering of the Tryptic Peptides of CNBr Fragment II_3: Characterization of the Chymotryptic and Thermolytic Peptides. The amino acid composition of the chymotryptic peptides isolated from CNBr fragment II_3 is given in Table III. All but seven out of the total 69 residues could be accounted for, including the five arginines. Thus, all the overlap peptides were found except for the one containing aminoethylcysteine which was obtained separately from a thermolytic digest of CNBr fragment II_3.

C_1 was an acidic, ninhydrin-negative peptide with a composition and mobility at pH 6.5 identical to $(T_0)C_1$. *C_2* was a ninhydrin-negative peptide which includes residues 1-11. Since it had a negative charge of minus 2 at pH 6.5, one of the Glx residues must be Gln. *C_3* was a basic peptide at pH 6.5 which links T_0 to T_1. Its sequence was determined by dansyl Edman degradation. Because of its mobility, residue 16 must have been Gln. *C_4* spanned C_2 and C_3 and provided an overlap between T_0 and T_1. Despite its relatively low yield, it was subjected to dansyl Edman degradation and provided information for the assignment of residues 5 through 10. *C_5* had a composition and COOH-terminal sequence consistent with its assignment as the NH_2-terminal portion of T_2. *C_6*, Asp-Tyr, represented residues 32 and 33, as can be seen in Figure 4. *C_7* provided a crucial overlap between T_2, T_3, and T_4. The order of these peptides was confirmed by the sequence of the first seven residues of C_7. The COOH-terminal tryptophan released by CPA provided positive evidence that this residue occupied position 47 in the sequence of V_H New. *C_8*, Ile-Gly-Tyr, together with the last four residues of C_7 account for the composition of $(T_4)th_1$. *C_9*, Val-Phe-Tyr, together with C_{10} accounts for $(T_4)th_2$. *C_{10}* was a decapeptide. Eight steps of dansyl Edman degradation provided a sequence which filled in positions 54-62. *C_{11}*, provided the evidence to connect T_4, T_5, and T_6 in that order. *C_{12}* occupied positions 19 and 20.

Thermolytic peptides were isolated to provide an overlap between T_1 and T_2 and to clarify and to confirm other regions of CNBr fragments II_3. With the exception of th_1 and th_3, only their amino acid compositions were obtained. These are given

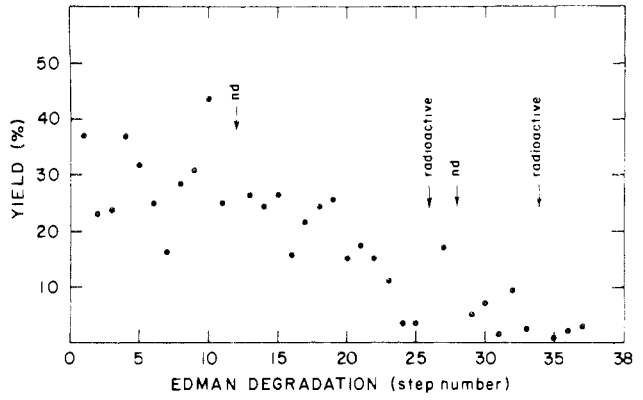

FIGURE 6: A plot of the yield vs. step number for the automatic Edman degradation of the CNBr fragment spanning residues 70–249 (peak A, Figure 2B); nd, residue not determined. An aliquot of each fraction was counted and the step where radioactivity was found is indicated on the graph. The radioactivity represents a carboxymethylcysteine residue.

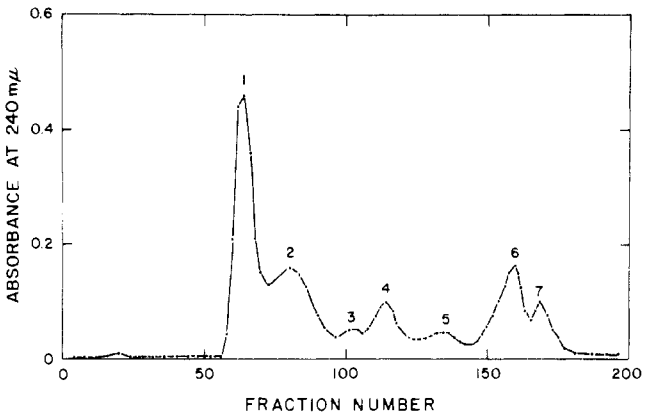

FIGURE 7: Gel filtration of the tryptic peptides from succinylated CNBr fragment 2 (residues 70–249; peak B, Figure 2B). A 2.2 × 120 cm column of Sephadex G-75 equilibrated with 0.1 M NH_4HCO_3 was used. Fractions of 5 mL were collected and pooled as shown.

in Table IV and are consistent with the assignments shown in Figure 4. Th_2 confirms the linkage between T_0 and T_1, while th_3 gave the missing connection between T_1 and T_2.

This completed the sequence determination of residues 1–69.

Sequence Determination of Residues 70–125. The material represented by peak A (Figure 2B) was first checked for purity by dansyl Edman degradation. A clean, unique sequence, Leu-Val-Asp-Thr, was obtained. Because peak A was obtained by reduction of the disulfide bridge connecting the CNBr fragment analogous to fragment II_3 (residue 1–69) with the adjacent CNBr fragment (presumably comprising residues 70–249), it was considered to have been derived from the variable region of New heavy chain. For this reason, it was submitted to automatic Edman degradation. The following sequence was obtained:

$$\overset{70}{\underset{\text{Leu}}{}}\text{-Val-Asp-Thr-Ser-Lys-Asn-Gln-Phe-Ser-}\overset{80}{\underset{\text{Leu}}{}}\text{-Arg-Leu-}$$

$$\text{Ser-Ser-Val-Thr-Ala-Ale-Asp-}\overset{90}{\underset{\text{Thr}}{}}\text{-Ala-Val-Tyr-Tyr-Cys-}$$

$$\text{Ala-Arg-Asn-Leu-}\overset{100}{\underset{\text{Ile}}{}}\text{-Ala-Gly-Cys-Ile-Asp-}\overset{106}{\underset{\text{Val}}{}}\text{-}$$

The yields for each step are shown in Figure 6. After the 30th step, the yields dropped below 10%, so we decided to isolate the

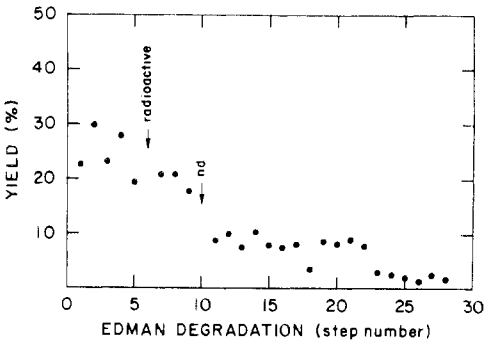

FIGURE 8: A plot of the yield vs. step number for the automatic Edman degradation of peak 1 (Figure 7), a tryptic peptide spanning residues 98–214 from succinylated CNBr fragment 2 (residues 70–249); nd, residue not determined. The step which released radioactivity is indicated and corresponds to [^{3}H]carboxymethylcysteine.

region that starts with residue 98 by treating CNBr fragment 2 (residues 70–249) (peak A Figure 2B) with [^{14}C]succinic anhydride and then digesting the material with trypsin. When this was done, and the digest filtered through Sephadex G-75, the material eluting close to the void volume (Figure 7, peak 1) turned out to be the peptide of interest. It was submitted to automatic sequencing with the following results:

$$\text{Asn-Leu-}\overset{100}{\underset{\text{Ile}}{}}\text{-Ala-Gly-Cys-Ile-Asp-Val-Trp-Gly-Gln-}\overset{110}{\underset{\text{Gly}}{}}\text{-}$$

$$\text{Ser-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-}\overset{120}{\underset{\text{Thr}}{}}\text{-Lys-Gly-Pro-}$$

$$\text{Ser-Val-}$$

The relative yields are given in Figure 8. Since the sequence of this peptide extends into the constant region (which begins at about residue 118, this completes the description of the sequence determination of V_H New. Finally, in Figure 9 the V_H New sequence is compared with a prototype human V_H sequence from subgroup II to which V_H is most closely related and to the V_H sequence of protein MOPC-315 a mouse myeloma protein that exhibits high-affinity binding of the 2,4-dinitrophenyl group. Inferences which can be drawn from these comparisons are presented in the Discussion.

Discussion

The amino acid sequence of the heavy-chain variable region of protein New was determined so that a three-dimensional model of protein New could be built from the 2-Å electron-density map (Poljak et al., 1974). In addition, since the number of human immunoglobulin V_H regions where the sequence has been completely established is small (~15), the results obtained with V_H New will add to the existing knowledge about the extent and pattern of sequence diversity exhibited by human V_H regions. The determination of the amino acid sequence of V_H New was a technically difficult project, mainly due to the low solubility of peptides which included residues 23 to 36 and 44 to 64 and which define antigen complementary regions (Poljak et al., 1973; Amzel et al., 1974). A further problem was that the peptides containing residues 5 and 6 (Glx-Glx) were in such short supply that we were not able to assign the amide unequivocally, although it was clear from the mobility of the relevant peptides that one of the two Glx residues must be Gln. Examination of the x-ray crystallographic model of New, however, shows that position 6 is occupied by a side chain that is at least partially internal, whereas position 5 has a side chain

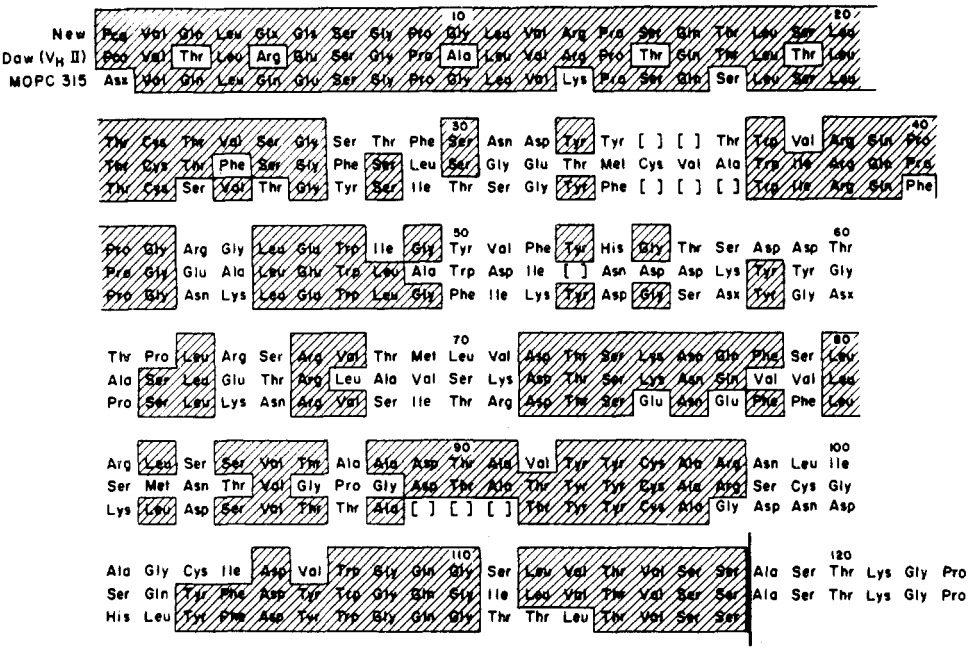

FIGURE 9: A comparison of the V_H regions of proteins New, Daw (which is representative of the V_HII subgroup) and MOPC-315 which is a mouse myeloma protein that has affinity for the 2,4-dinitrophenyl group. Identical residues, occupying the same positions when comparing any 2 of the 3 proteins shown here, are enclosed and shaded. The empty brackets indicate gaps that have to be introduced in the sequence in order to maximize the degree of homology among the proteins. The numbering is based on the New sequence and does not include the gaps. The proposed junction between the V_H and C_H1 region is indicated by the double vertical line between residues 117 and 118.

exposed to the surface. This would suggest that position 5 is glutamic acid and position 6 glutamine.

In spite of these difficulties, the primary structure proposed here is complete except for the amide assignment mentioned above and is totally consistent with the 2-Å electron-density map of this portion of protein New. Many other peptides and subpeptides that were obtained by digestion with pepsin, chymotrypsin, and thermolysin are not indicated here for reasons of brevity and redundancy, but they are also in agreement with the sequence given in Figure 4 and provide confirming evidence for the proposed structure.

A comparison of the V_H region sequence of protein New with other human V_H region sequences clearly shows that V_H New belongs to the V_HII subgroup of human myeloma immunoglobulins. The detailed comparison with one of the known V_HII regions (from protein Daw) (Figure 9) shows that 59 out of 117 residues are identical in both proteins. Of the remaining 58 residues, 42 can be accommodated by a single-base change. The regions of identity are clustered rather than being distributed randomly along the chain. For instance, of the first 26 residues 20 are the same. Other clusters occur between residues 36 and 47, where there are 9 identical amino acids; between residues 89 and 97, where all but one position are identical; and the span covering residues 105 to 117, where 11 out of 13 amino acids are the same. In addition there is a 6-residue stretch (72–77) of complete congruence. In order to maximize the number of identical residues between the two proteins, it was necessary to introduce a gap of 2 residues (positions 36, 37) in protein New and a gap of one residue (position 53) in protein Daw (V_HII). These gaps (or additions in the other proteins being compared) occur in regions where there are no matching residues, no matter how the sequences are lined up (Figure 9). When V_H New is compared to V_HII sequences in other human myeloma immunoglobulins besides protein Daw, additional gaps have to be introduced in New starting at positions 53 and 105 to maximize homology with proteins; Ou (Putman et al., 1971), Cor (Press and Hogg, 1970), and He (Cunningham et al., 1971). These regions correspond to those parts of the protein exhibiting "hypervariability" (Wu and Kabat, 1970; Kehoe and Capra, 1971) and to the segments of V_H New sequence which contribute to the antigen-binding site, namely, residues 28–33, 50–60, and 99–105.

Residues 39 (Gln), 45 (Leu), 47 (Trp), 94 (Tyr), and 107 (Trp) make contact with residues in the V_L regions (Poljak, 1975). Because of this, one might expect them to be identical or to be replaced with amino acids of similar structure in other V_H regions. This is indeed the case, not only for proteins that belong to the V_HII subgroup but also for V_H regions of all human immunoglobulins. Even when V_H regions from immunoglobulins from other species are examined, residues in these positions are almost always constant. A striking exception is protein Ou in which Arg, rather than Gln, has been reported to occupy position 39 (Putman et al., 1971). Given that all other V_H regions which have been sequenced through this residue have Gln, it would suggest that the sequence of protein Ou would bear reinvestigation in this region. Since the V_L contact residues are constant or nearly constant, it has been proposed (Poljak, 1975) that the interaction between V_H and V_L is independent of H- and L-chain subgroup sequence variation, so that any L chain, regardless of class, has the potential to pair with any H chain. Without any apparent structural basis for restriction on pairing of L and H chains, it is possible then to make use of all the information contained in the structural genes for H and L chains in such a way as to maximize the extent of diversity with respect to antigen binding.

In addition to the residues just discussed, most of the nonpolar, hydrophobic amino acid side chains placed between the two β sheets which make up the backbone of the V_H sequences are invariant or almost invariant. These are: Leu-4, Leu-18, Leu-20, Cys-22, Tyr-34, Trp-36, Ile-48, Val-67, Met-69, Phe-78, Leu-80, Leu-82, Val-85, Ala-91, Tyr-93, Cys-95, Val-106, Ser-111, Val-113, and Val-115. With regard to the

boundary between V_H and C_H1, sequence comparisons alone are not sufficient to precisely pinpoint the V/C bridge, but examination of the three-dimensional structure clearly shows that the Val-Ser-Ser sequence, which is shared by γ and μ chains (Florent et al., 1974), is the end of the V_H region and, after a sharp bend, Ala marks the beginning of the C_H1 homology subunit (Poljak, 1975). Finally, it is worth noting the remarkable degree of identity and homology between protein New and a mouse myeloma protein MOPC 315 (Figure 9) (Francis et al., 1974). The same number of identical residues (59 out of 117) is found when comparing New with MOPC 315, as was observed in the comparison of New with V_HII (Daw). Of the remaining 58 residues which do not match, 36 can be explained by assuming that a single-base change is responsible for the amino acid replacement. As in the comparison between New and V_HII, the gaps and amino acid substitutions (New vs. MOPC 315) not consistent with single-base changes are also located in the hypervariable regions. Two gaps are required in the MOPC 315 sequence to get an optimal fit. In certain positions (28, 36, 48, 58, 62, 92, 103, and 104), where there is a lack of identity between New and MOPC 315, there is identity between MOPC 315 and V_HII (Daw). All of these comparisons are consistent with the notion that the structural genes specifying these V_H regions were derived from a common precursor gene. This idea has been proposed previously (Hood et al., 1975) to account for other similar situations in V_L and V_H regions of immunoglobulins from the same as well as from different species.

Correlations between the amino acid sequence and the three-dimensional structure of V_H and Fab New which have been discussed in detail before (Poljak et al., 1973, 1974) but with incomplete data, will be reexamined in the near future using information derived from the refinement of the x-ray crystallographic model and from the complete V_H sequence reported here. This information, relating amino acid sequence to three-dimensional structure, will be important in precisely defining the chemical contacts and interactions between the combining site of IgG New and its ligands, such as vitamin K_1-OH and carminic acid (Richards et al., 1975; Amzel et al., 1974). It is hoped that a more complete characterization of this system will serve as a general model for antibody–hapten interactions.

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