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The Covalent Structure of a Human γG-Immunoglobulin. I. Isolation and Characterization of the Whole Molecule, the Polypeptide Chains, and the Tryptic Fragments*

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ABSTRACT: A human γG-immunoglobulin (Eu) has been purified from the plasma of a patient with multiple myeloma in order to provide material for amino acid sequence analysis of the entire molecule. Eu and its constituent heavy and light chains have been characterized by ultracentrifugal and immunological analyses, and their amino acid compositions and end groups have been determined. The molecular weight of the intact molecule (154,000) is consistent with the presence of two light chains (mol wt 23,500) and two heavy chains (mol wt 51,600). Amino acid analyses and end group determinations on Eu and the isolated chains also support this conclusion. Limited tryptic digestion of partially reduced and alkylated Eu produced fragments similar to the Fab and Fc fragments produced by papain digestion.

Structural studies have shown that immunoglobulins are multichain proteins (Edelman, 1959; Edelman and Poulik, 1961) made up of a four-chain unit containing two light and two heavy polypeptide chains (Fleischman *et al.*, 1962; Edelman and Benacerraf, 1962; Fougereau and Edelman, 1965). It is currently believed that the information for the specificity of antibodies resides in the amino acid sequences of light and heavy chains (Haber, 1964; Whitney and Tanford, 1965) and that the range of specificities is reflected in a large variety of sequences. Normal immunoglobulins are therefore heterogeneous, and it is difficult to carry out amino acid sequence analysis even on preparations of highly purified antibodies to a single hapten antigen. In the disease multiple myeloma, plasma cell tumors may be found which produce homogeneous immunoglobulins having the characteristic multichain structure (Edelman and Poulik, 1961; Edelman and Gally, 1962). Following the demonstration that urinary Bence–Jones proteins from patients with multiple myeloma are homogeneous light chains corresponding to those found in serum myeloma proteins (Edelman and Gally, 1962; Schwartz and Edelman, 1963), sequence studies have been carried out on

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Bence-Jones proteins in a number of laboratories (Hilschmann and Craig, 1965; Titani *et al.*, 1965; Milstein, 1966; Hilschmann, 1967). Bence-Jones proteins from plasma cell tumors in mice have also been extensively studied, and partial sequences of several mouse tumor proteins have been published (Hood *et al.*, 1966; Gray *et al.*, 1967). Studies on the sequence of heavy chains from both human pathological (Press *et al.*, 1966a,b) and rabbit normal (Hill *et al.*, 1966; Givol and Porter, 1965; Wilkinson *et al.*, 1966; Cebra, 1967) immunoglobulins have also been reported. In no case, however, has the entire sequence of a γG-immunoglobulin or myeloma protein been analyzed. The complete amino acid sequence of a myeloma protein would serve as a valuable reference for sequence studies on other immunoglobulins.

This paper is the first in a series on the covalent structure of a human myeloma protein (Eu). It describes the purification and characterization of Eu, its polypeptide chains, and the fragments produced by limited tryptic digestion. Subsequent papers in this series will describe the properties and ordering of fragments produced by cleavage with cyanogen bromide, proof of the gross arrangement of the chains and position of interchain disulfide bonds, and the amino acid sequences of the light and heavy chains.

Materials and Methods

Preparation of Purified Eu Myeloma Protein. Plasma collected in sterile polyethylene bags was mixed with an equal volume of 68% saturated ammonium sulfate at 4°. The mixture was immediately centrifuged at 400*g* for 15 min to remove the precipitate, which was dissolved in water, mixed with an equal volume of 68% saturated ammonium sulfate, and centrifuged. The original supernatant was mixed with an equal volume of 68% saturated ammonium sulfate to increase the yield of myeloma protein. These precipitates were enriched in myeloma protein and could be conveniently stored at 4°. Moderate amounts of Eu were prepared from the precipitate by zone electrophoresis on starch (Kunkel, 1954), but gram quantities were more easily prepared by ion-exchange chromatography on DEAE-cellulose (Peterson and Sober, 1956; Fahey and Horbett, 1959). Cellex D (Bio-Rad Laboratories, Richmond, Calif., exchange capacity 0.98 mequiv/g) was equilibrated with 0.01 M potassium phosphate buffer (pH 8.4) and poured into a 5.5 × 150 cm column. About 250 ml of a 5% solution of the ammonium sulfate precipitate which had been dialyzed against the phosphate buffer was loaded on the column and eluted with the same buffer. Eu emerged from the column in high yield, while the other components of the precipitate were retained on the ion exchanger. About 70% of the protein in the precipitate was recovered as myeloma protein.

Partial Reduction and Alkylation. In some experiments, a 2-3% solution of protein in 0.15 M Tris, 0.15 M in NaCl, 0.002 M in EDTA (pH 8.0) was made 0.01 M in dithiothreitol (Cleland, 1964; Calbiochem, Los Angeles, Calif.) and allowed to stand at room temperature for 2 hr. Iodoacetamide (K & K Laboratories, Plainview, N. Y., twice recrystallized from water) was added to a final concentration of 0.04 M, and the solution was kept in the dark for 15 min. In other experiments, a 30-fold M excess of dithiothreitol over the protein was added (final dithiothreitol concentration about 0.003 M), and after 40 min, a 60-fold M excess of iodoacetamide was added.

Reagents were removed by dialysis against water in the dark at 4°. In order to separate chains directly, the solution was applied to a Sephadex column which was wrapped in aluminum foil to shield the sample from light.

Preparation of Chains. Heavy and light chains were prepared from partially reduced and alkylated Eu by gel filtration on Sephadex G-100 in 1 M propionic acid (Fleischman *et al.*, 1962). For analytical purposes, 5–20 mg of partially reduced and alkylated protein was loaded on a 1 × 100 cm column. Larger amounts of chains were prepared on 4.5 × 150 cm columns with loads of 0.5–1 g. Heavy- and light-chain fractions were further purified by repeated gel filtration under the same conditions.

For the determination of total half-cystine and methionine content, chains were prepared by reduction in 0.01 M dithiothreitol and gel filtration in 1 M propionic acid which was 0.002 M in dithiothreitol.

Preparation of Tryptic Fragments. Partially reduced and alkylated Eu (500 mg) was dissolved in 10 ml of 0.15 M NaCl which was 0.05 M in $CaCl_2$. The solution was placed in a water-jacketed vessel at 25°, and the pH was adjusted to 8.0. Trypsin (10 mg) treated with L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK-trypsin, Calbiochem, Los Angeles, Calif., lot 65345) was added, and the pH was maintained at 8.0 by the automatic addition of 0.1 N NaOH (Radiometer TTT1b titrator). After 1-hr digestion, 20 mg of soybean trypsin inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio, lot 8822, five-times recrystallized) was added. The sample was chilled at 4° and was subjected to starch zone electrophoresis to separate the fragments.

Tryptic fragments were also separated by ion-exchange chromatography on DEAE-cellulose (Cellex D, Bio-Rad Laboratories, Richmond, Calif., exchange capacity 0.98 mequiv/g). In a typical fractionation, 5.5 g of digest was dialyzed against 0.005 M sodium phosphate (pH 8.5) and applied to a 3 × 35 cm column of Cellex D equilibrated with the same buffer. Fab(t) was not retarded by the column, and Fc(t) was eluted with a linear gradient from 1 l. of the initial buffer to 1 l. of the same buffer made 0.15 M in NaCl.

Preparation of the Papain Fragments. Digestion with papain (Worthington Biochemical Corp., Freehold, N. J., lot PAP 5593, twice crystallized) was performed as described by Porter (1958), except that digestion was carried out for 5 min. The fragments were separated by zone electrophoresis on starch.

Ultracentrifugation. A Spinco Model E ultracentrifuge equipped with interference and phase-plate schlieren optics and automatic temperature control was used. Sedimentation velocity experiments were

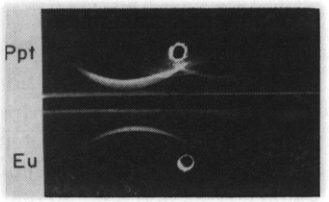

FIGURE 1: Immunoelectrophoresis of the 34% ammonium sulfate fraction of Eu plasma (precipitate, upper well) and purified Eu (lower well). Rabbit antiserum against human serum was used.

done at 52,000 rpm in double-sector cells with sapphire windows. Molecular weights were determined by the high-speed equilibrium method of Yphantis (1964). Molecular weight data are presented as graphs of the logarithm of the concentration expressed in microns of fringe deviation *vs.* the radius (r) squared divided by two, and the weight-average molecular weights were calculated from the least-squares slope of the line through the points. Molecular weight heterogeneity is reflected by consistent deviations from linearity in the graph. Partial specific volumes, calculated from the amino acid composition (McMeekin *et al.*, 1949), were 0.73 cc/g for Eu, its constituent chains, and the tryptic fragments.

Uncertainty in the molecular weight due to errors in the determination of the concentration distribution in the cell was calculated to be 5%, using the procedure described by Yphantis (1964). Uncertainty in the calculated partial specific volume would introduce further errors, estimated to be less than 5%.

Amino Acid Analysis. Samples were hydrolyzed in metal-free 6 N HCl at 110° for 20, 48, or 72 hr in evacuated, sealed, Pyrex tubes. Quantitative analyses were performed by the technique of Spackman *et al.* (1958) with minor modifications, using a Beckman Model 120C amino acid analyzer equipped with 6.6-mm flow cells, a 1-mV range recorder, and an Infotronix (Houston, Texas) Model CRS-11AB integrator. Acidic and neutral amino acids were determined on a 57-cm column of Beckman AA-15 resin, with a buffer flow rate of 93 ml/hr using the standard 0.2 N sodium citrate buffers at pH 3.28 and 4.25. Basic amino acids were determined on a 4.8-cm column of Beckman PA-35 resin using 0.35 N sodium citrate buffer containing 5% (v/v) *n*-propyl alcohol (Hubbard, 1965) with a flow rate of 93 ml/hr. The columns were maintained at 50°. The analyzer was modified by adding a second ninhydrin pump and reaction coil so that the short column could be used continuously. The original suppressed 570-mμ channel, equipped with a 6.6-mm flow cell, was used exclusively for the short column. Analyses at the 10-nmole level were reproducible within 5%.

Methionine and half-cystine values were determined on unalkylated samples by the performic acid oxidation method of Moore (1963). Tryptophan content was estimated from the tyrosine to tryptophan ratio determined by the spectrophotometric method of Goodwin and Morton (1946).

End-Group Analyses. Quantitative end-group analysis was performed by the carbamylation method of Stark and Smyth (1963). Qualitative end-group analysis was done by the dansyl[1] method of Gray (1967), except that the dansylamino acids were separated on polyamide thin layers (Woods and Wang, 1967).

Other Techniques. Immunoelectrophoresis was done using the technique of Scheidegger (1955). Immune diffusion was carried out as described by Olins and Edelman (1962). Starch gel electrophoresis in formate–urea was performed as described by Edelman and Poulik (1961). The protein concentration of starch zone eluates was determined by a modified Folin–Ciocalteu method (Lowry *et al.*, 1951). Hexose was determined with anthrone reagent using a mixed standard of galactose:mannose:fucose (4.5:4.5:1) (Müller-Eberhard and Kunkel, 1956). Hexosamine was determined by a modification of the method of Elson and Morgan (1933), as described by Müller-Eberhard and Kunkel (1956).

Results

Characterization of Eu Myeloma Protein. Eu obtained from the ammonium sulfate precipitate of plasma by starch zone electrophoresis or DEAE-cellulose chromatography was free of other plasma proteins, as determined by antigenic analysis. Figure 1 shows an immunoelectrophoresis of the 34% ammonium sulfate fraction of Eu plasma and purified Eu, using rabbit antiserum against whole human serum. Purified Eu gave a sharp line of precipitate characteristic of a myeloma protein, rather than the broad arc of normal γG-immunoglobulin. No contamination with other serum proteins was observed. Genetic typing of Eu, carried out by Dr. H. H. Fudenberg, showed it to be Gm (a−, f+, b−) and Inv (a−). This suggests that the Eu heavy chains belong to subgroup γG1.

In the analytical ultracentrifuge, purified Eu sedimented as a single, symmetrical peak with an $s^0_{20,w}$ of 6.6 S, in 0.05 M Tris–0.15 M NaCl (pH 8.0). The molecular weight of Eu, determined by the high-speed sedimentation equilibrium method of Yphantis (1964), is shown in Figure 2 in terms of a plot of the logarithm of the protein concentration *vs.* the radius squared divided by two. The data points fit a straight line and no curvature is apparent, suggesting that the preparation is homogeneous with respect to molecular weight. The weight-average molecular weight of the preparation was 154,000 ± 8000.

Hexose and hexosamine determinations showed that the carbohydrate content of Eu was similar to that of pooled human γG-immunoglobulin (Clamp and Putnam, 1964). On this basis, the molecular weight of the carbohydrate component was estimated to be 4000, and the calculated molecular weight of the polypeptide component was 150,000.

The amino acid compositions of 20-, 48-, and 72-hr hydrolysates of Eu were calculated on the basis of

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

TABLE I: Amino Acid Composition of Eu Myeloma Protein.

	Residues/150,000 g			
	20 hr	48 hr	72 hr	Av[a]
Lys	92.2	92.8	90.6	92.0
His	24.3	23.0	23.5	23.6
Arg	33.8	34.8	34.5	34.3
Asp	99.3	98.3	99.0	98.9
Thr	105	106	98.4	112
Ser	155	130	132	166
Glu	146	145	146	146
Pro	102	105	103	103
Gly	94.1	95.0	97.3	95.5
Ala	71.3	72.5	74.8	72.9
Cys[b]	33.9			33.9
Val	118	123	129	129
Met[b]	17.7			17.7
Ile	30.8	34.3	32.2	33.2
Leu	93.4	94.6	94.6	94.2
Tyr	51.8	54.2	54.4	53.5
Phe	46.3	46.6	47.7	46.9
Trp	22.4			22.4

[a] Threonine values are based on a linear extrapolation to zero hydrolysis time. Serine values are based on a linear extrapolation of 20- and 48-hr values to zero time. Valine values are from the 72-hr hydrolysate. The 20-hr value for isoleucine is not included in the average. [b] Half-cystine and methionine values are averages of three analyses from 20-hr hydrolysates of oxidized samples.

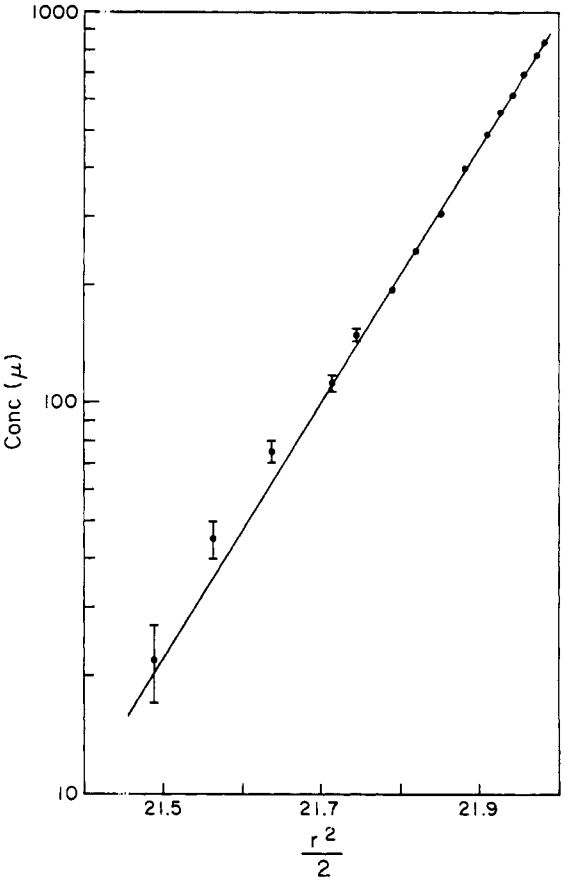

FIGURE 2: Plot of the logarithm of the concentration *vs.* (radius)²/2 used for the determination of the molecular weight of Eu. Initial concentration 0.12 mg/ml in 0.05 M Tris, 0.15 M in NaCl (pH 8.0); equilibrium speed 20,000 rpm. Concentrations are expressed in microns of fringe deviation. Bars indicate the estimated standard deviation (5 μ) of each point.

residues/150,000 g, and the results are given in Table I. For amino acids which are stable to acid hydrolysis, the average of the three values was taken. Threonine values are based on a linear extrapolation to zero hydrolysis time. The serine content decreased between 20 and 48 hr, and then increased slightly at 72 hr. The value given is an estimate obtained by a linear extrapolation of the 20- and 48-hr values to zero hydrolysis time.

Preparation and Characterization of Eu Heavy and Light Chains. Light and heavy chains were prepared from Eu by partial reduction at pH 8 with dithiothreitol, alkylation with iodoacetamide, and separation on Sephadex G-100 in 1 M propionic acid; a typical analytical chromatogram is shown in Figure 3. The small peak eluted first from the column appeared to contain aggregated material, as tested by starch gel electrophoresis in urea. The second peak, designated H, accounted for 65% of the material loaded and contained the heavy chains. The final peak (L) accounted for 30% of the material loaded and contained the light chains. In most cases, the heavy- and light-chain fractions were further purified by repeated gel filtration under the same conditions. Light and heavy chains prepared by this method were free of cross-contamination as determined by starch gel electrophoresis in formate–urea.

The molecular weights of the chains were measured in 20% acetic acid containing 0.5% sucrose to provide a stabilizing density gradient (see Yphantis, 1964). A graph of concentration *vs.* the radius squared divided by two for Eu heavy chain is shown in Figure 4. The line is straight, consistent with homogeneity of the preparation, and the weight-average molecular weight of the preparation was 51,600 ± 2600. A similar plot for the light chain is shown in Figure 5. The line is slightly curved, and the point weight-average molecular weights (see Yphantis, 1964) tend to decrease as the concentration increases. The reciprocal of the reduced point weight-average molecular weights plotted against the concentration at each point is shown in Figure 5 (insert). The plot is linear, and the least-square line through the points has an intercept corresponding to a molecular weight of 23,500. The molecular weight of the light chain was taken to be 23,500 ± 1200. The molecular weight of Eu, calculated from the sum of the molecular weights of the chains, is 150,200, which is within 3% of the observed molecular weight of 154,000.

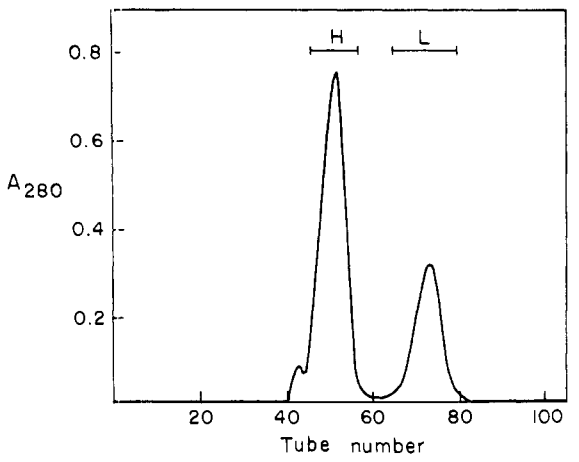

FIGURE 3: Separation of light and heavy chains from partially reduced and alkylated Eu (5 mg) by gel filtration on Sephadex G-100 in 1 M propionic acid. Column dimensions, 1 × 100 cm; volume per tube, 0.83 ml. H = heavy chains, L = light chains, A_{280} = absorbance of effluent at 280 mμ.

The amino acid compositions of the heavy and light chains were calculated on the basis of the observed molecular weights, assigning all of the carbohydrate to the heavy chains. The results are given in Tables II and III. Total half-cystine and methionine values were measured by performic acid oxidation of chains prepared without alkylation. Light chains prepared by alkylation with iodoacetamide contained 1 mole of *S*-carboxamidomethylcysteine/mole, and heavy chains contained 3 moles/mole.

End-Group Analysis of Eu and the Isolated Chains. End-group analysis of Eu by the carbamylation method gave 1.7 moles of aspartic acid/mole of protein. Qualitative end group analysis of the isolated light chain by the dansyl method gave only aspartic acid; an amino-terminal residue was not detected for the heavy chain. These data are consistent with the presence in the molecule of two heavy chains with blocked amino termini and two light chains which contribute the 2 moles of aspartic acid/mole of myeloma protein.

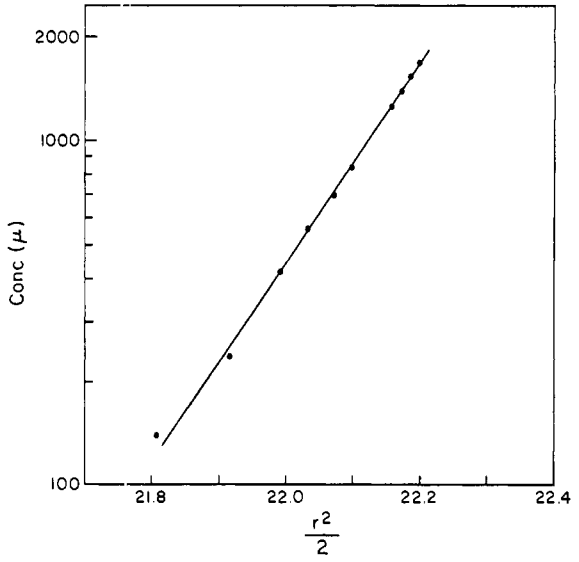

FIGURE 4: Plot of the logarithm of the concentration *vs.* (radius)²/2 used for the determination of the molecular weight of Eu heavy chain. Initial concentration, 0.67 mg/ml in 20% acetic acid, 0.5% sucrose; equilibrium speed 32,000 rpm. Concentrations are expressed in microns of fringe deviation.

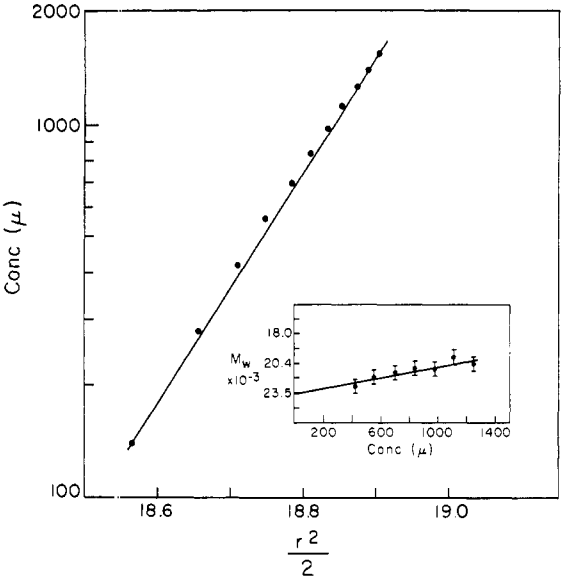

FIGURE 5: Plot of the logarithm of the concentration *vs.* (radius)²/2 used for the determination of the molecular weight of Eu light chains. Initial concentration, 0.75 mg/ml in 20% acetic acid, 0.5% sucrose; equilibrium speed 52,000 rpm. Insert: reciprocal point weight-average molecular weights of light chains as a function of concentration. Concentrations are expressed in microns of fringe deviation.

Tryptic Fragments of Partially Reduced and Alkylated Eu. Because of its restricted specificity, trypsin, rather than papain, was used for the preparation of fragments from Eu. The highest yields of fragments were obtained by digesting partially reduced and alkylated Eu in 0.15 M NaCl–0.05 M $CaCl_2$ at pH 8.0. After the addition of trypsin (Figure 6) about 4 moles of base/mole of protein was taken up in the first 5 min. The rate of base uptake dropped steadily, and after

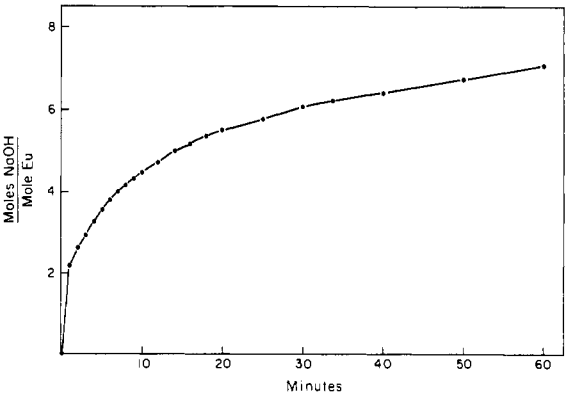

FIGURE 6: Base uptake during tryptic digestion of partially reduced and alkylated Eu. The protein (500 mg) was dissolved in 10 ml of 0.15 M NaCl, 0.05 M in $CaCl_2$, at 25°, and the pH was maintained at 8.0 by the addition of 0.1 N NaOH.

TABLE II: Amino Acid Composition of Eu Heavy Chain.

	Residues/47,600 g			
	20 hr	48 hr	72 hr	Av[a]
Lys	30.4	32.1	30.8	31.1
His	8.2	8.9	9.3	8.8
Arg	10.2	11.0	10.2	10.5
Asp	31.6	31.9	31.7	31.7
Thr	32.5	32.6	31.3	33.3
Ser	44.7	29.2	38.0	56.0
Glu	44.2	44.8	44.0	44.3
Pro	38.3	38.5	39.1	38.6
Gly	32.0	33.4	33.0	32.8
Ala	22.0	22.4	22.0	22.1
Cys	11.8			11.8
Val	41.6	45.0	46.5	46.5
Met	5.9			5.9
Ile	8.2	9.6	9.5	9.5
Leu	29.8	30.0	30.9	30.2
Tyr	18.6	18.8	17.2	18.7
Phe	16.2	16.5	15.5	16.1
Trp	8.3			8.3

[a] Threonine values are based on a linear extrapolation to zero hydrolysis time. Serine values are based on a linear extrapolation of 20- and 48-hr values to zero time. Isoleucine and valine values are from the 72-hr hydrolysate. Half-cystine and methionine values are from 20-hr hydrolysates of oxidized samples of heavy chains prepared without alkylation. The 72-hr value for tyrosine is not included in the average.

TABLE III: Amino Acid Composition of Eu Light Chain.

	Residues/23,500 g			
	20 hr	48 hr	72 hr	Av[a]
Lys	15.0	14.8	14.2	14.7
His	3.1	3.0	2.4	3.0
Arg	5.2	5.0	5.3	5.2
Asp	16.2	16.4	16.6	16.4
Thr	16.9	16.5	16.3	17.2
Ser	27.3	24.6	22.7	29.1
Glu	24.0	24.6	25.1	24.6
Pro	11.3	11.7	12.4	11.8
Gly	14.2	14.3	14.2	14.2
Ala	13.2	13.0	13.0	13.1
Cys	5.2			5.2
Val	15.3	16.4	16.9	16.9
Met	2.9			2.9
Ile	5.8	5.9	6.0	6.0
Leu	14.9	15.0	15.1	15.0
Tyr	8.4	8.7	8.9	8.7
Phe	7.7	8.0	8.1	7.9
Trp	3.0			3.0

[a] Threonine and serine values are based on a linear extrapolation to zero hydrolysis time. Isoleucine and valine values are from the 72-hr hydrolysate. Half-cystine and methionine values are from 20-hr hydrolysates of oxidized samples of light chains prepared without alkylation.

60-min digestion about 7 moles of base had been taken up/mole of protein.

Electrophoresis of a 1-hr digest on starch (Figure 7) showed three components. The slowest moving (most cathodal) component, accounting for 63% of the protein, was immunologically identical with the Fab fragment produced by papain digestion as shown in Figure 8. The fastest moving component, accounting for 34% of the protein, was immunologically identical with the Fc fragment (Figure 8). The tryptic fragments were designated Fab(t) and Fc(t), respectively, to distinguish them from the similar products of papain digestion. A minor component which remained near the origin (Figure 7) accounted for about 3% of the digested protein. Its electrophoretic behavior on cellulose acetate was identical with that of undigested myeloma protein.

The molecular weights of Fab(t) and Fc(t) fragments were determined in 0.05 M Tris, 0.15 M in NaCl (pH 8.0). The graph of concentration *vs.* the radius squared divided by two for Fab(t) is shown in Figure 9. The weight-average molecular weight for the whole preparation was 46,000 ± 2300. A similar plot for Fc(t) (Figure 10) showed consistent deviation of the points at lower radii, thus suggesting the presence of a lower molecular weight component. The weight-average molecular weight of the largest component was estimated to be 48,000 ± 2400. The heterogeneity may be due to a population of Fc(t) fragments which are similar but not identical and which arise because of lack of specificity in the tryptic cleavage.

The amino acid compositions of the tryptic fragments based on molecular weights of 46,000 for Fab(t) and 48,000 for Fc(t) are given in Table IV together with the calculated composition of the whole protein based on the sum of one Fc(t) and two Fab(t) fragments per mole. The calculated composition agrees with the observed composition within 8%. These compositions and the yields after starch zone electrophoresis suggest that losses of large amounts of material due to extensive tryptic digestion do not occur in the preparation and isolation of the tryptic fragments.

Discussion

The isolation and characterization of the human γG myeloma protein Eu, its constituent polypeptide chains, and enzymatic fragments have been carried out to provide quantitative data for studies on the covalent structure of this protein.

The purity of the myeloma protein may be judged initially by its freedom from contamination with proteins other than γG-immunoglobulins, and finally by homogeneity of amino acid sequence. Eu was free

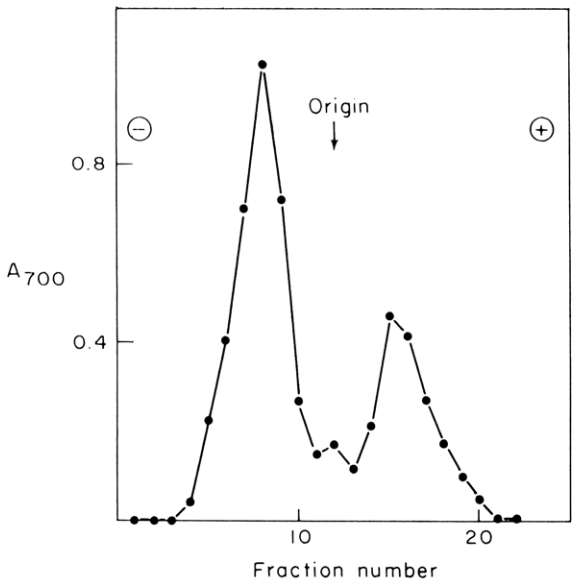

FIGURE 7: Zone electrophoresis on starch of a tryptic digest of partially reduced and alkylated Eu. Ordinate: protein concentration as determined with Folin's reagent, expressed as absorbance of the reaction mixture at 700 mμ (A_{700}). Abscissa: fraction number; (−) = cathode, (+) = anode.

of other serum proteins as determined by electrophoretic and immunologic criteria. Although Eu gave the relatively sharp electrophoretic bands typical of myeloma proteins, a small contamination with normal γG-immunoglobulins from the patient's serum cannot be excluded. This type of contamination would probably not lead to significant errors in sequence studies. A more serious consideration is that the myeloma protein may be composed of a small number of similar proteins which are indistinguishable by means other than actual sequence determination. The classical criterion of end-group analysis for establishing the homogeneity of a protein is not sufficient in this case, since the preparation could be composed of a small number of proteins with the same end group but differing in sequence. The yields of fragments, such as those resulting from specific chemical cleavage, become critical

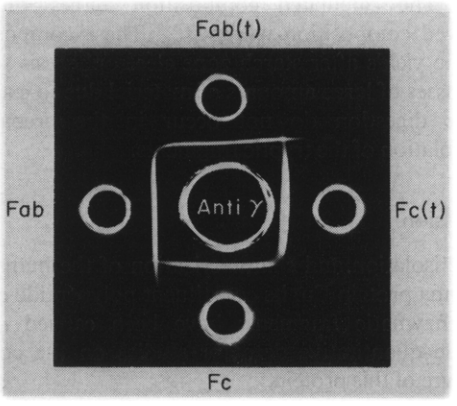

FIGURE 8: Comparison of tryptic and papain fragments of Eu by immunodiffusion. Fab(t), Fc(t) = tryptic fragments; Fab, Fc = papain fragments. Anti-γ = rabbit antiserum against human γ-globulin.

TABLE IV: Amino Acid Composition of Fab(t) and Fc(t) Fragments.[a]

	Fab(t) Residues/ 46,000 g	Fc(t) Residues/ 48,000 g	2 Fab(t) + Fc(t)	Eu Residues/ 150,000 g
Lys	29.2	33.1	91.5	92.0
His	4.0	13.7	21.7	23.6
Arg	9.1	11.3	29.5	34.3
Asp	30.6	39.3	100	98.9
Thr	37.4	29.9	105	112
Ser	59.3	36.4	155	166
Glu	45.7	50.9	142	146
Pro	26.8	44.0	97.6	103
Gly	38.8	21.6	99.2	95.5
Ala	29.6	16.4	75.6	72.9
Cys	7.8	9.4	25.0	33.9
Val	42.8	46.6	132	129
Met	6.0	5.7	17.7	17.7
Ile	11.9	8.7	32.5	33.2
Leu	29.3	34.0	92.6	94.2
Tyr	17.1	18.2	52.4	53.5
Phe	15.7	14.5	45.9	46.9
Trp	9.6	6.6	25.8	22.4

[a] Threonine and serine contents are based on a linear extrapolation of values from 20- and 72-hr hydrolysates to zero time. Isoleucine and valine values are from 72-hr hydrolysates. Cys is total recovered half-cystine plus *S*-carboxymethylcysteine.

TABLE V: Comparison of the Amino Acid Composition of Eu with That Calculated by Addition of the Compositions of Two Heavy and Two Light Chains.

	Light Chain Residues/ 23,500 g	Heavy Chain Residues/ 47,600 g	2(heavy + light)	Eu Residues/ 150,000 g
Lys	14.7	31.1	91.6	92.0
His	3.0	8.8	23.6	23.6
Arg	5.2	10.5	31.4	34.3
Asp	16.4	31.7	96.2	98.9
Thr	17.2	33.3	101	112
Ser	29.1	56.0	170	166
Glu	24.6	44.3	138	146
Pro	11.8	38.6	101	103
Gly	14.2	32.8	94.0	95.5
Ala	13.1	22.1	70.4	72.9
Cys	5.2	11.8	34.0	33.9
Val	16.9	46.5	127	129
Met	2.9	5.9	17.6	17.7
Ile	6.0	9.5	31.0	33.2
Leu	15.0	30.2	90.4	94.2
Tyr	8.7	18.7	54.8	53.5
Phe	7.9	16.1	48.0	46.9
Trp	3.0	8.3	22.6	22.4

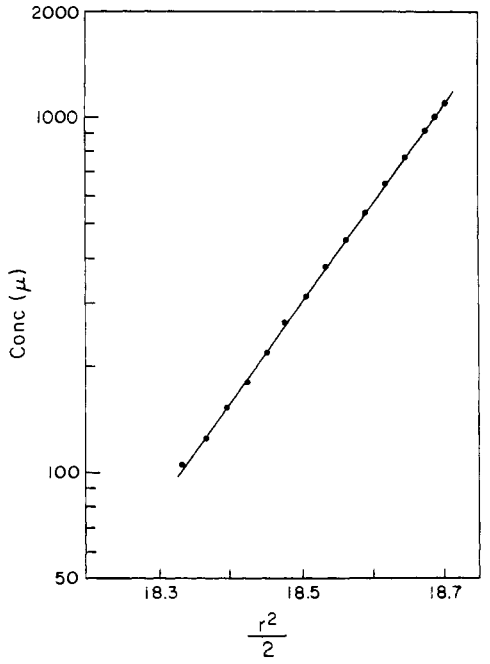

FIGURE 9: Plot of the logarithm of the concentration *vs.* (radius)²/2 used for the determination of the molecular weight of the Fab(t) fragment. Initial concentration, 0.15 mg/ml in 0.05 M Tris, 0.15 M in NaCl (pH 8.0); equilibrium speed 34,000 rpm. Concentrations are expressed in microns of fringe deviation.

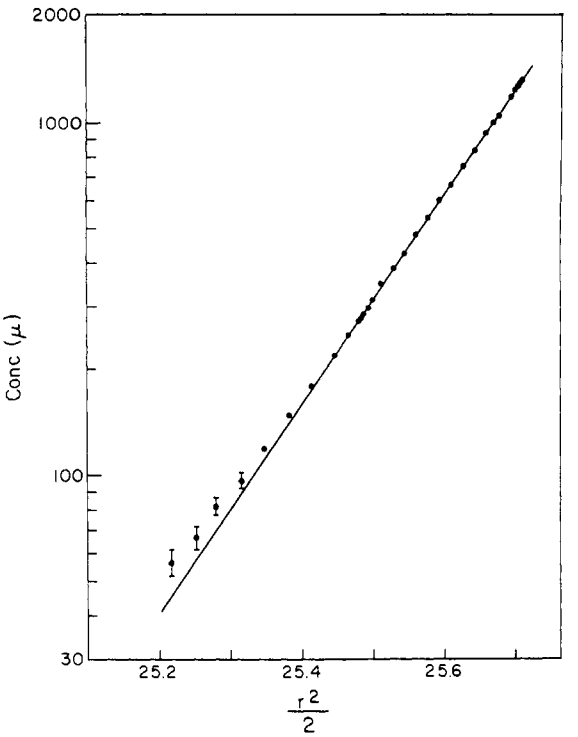

FIGURE 10: Plot of the logarithm of concentration *vs.* (radius)²/2 used for the determination of the molecular weight of the Fc(t) fragment. Initial concentration, 0.26 mg/ml in 0.05 M Tris, 0.15 M in NaCl (pH 8.0); equilibrium speed 34,000 rpm. Concentrations are expressed in microns of fringe deviation. Bars indicate the estimated standard deviation (5 μ) of each point.

in assessing the homogeneity of the myeloma protein from this point of view. Evidence for high yields of cyanogen bromide fragments is presented in a subsequent paper in this series (Waxdal *et al.*, 1968a).

Our data provide further evidence that the immunoglobulin molecule is made up of two heavy and two light chains (Edelman and Gally, 1962; Fleischman *et al.*, 1963; Fougereau and Edelman, 1965). Twice the sum of the molecular weights of the isolated chains agrees with the molecular weight of the intact molecule within experimental error. The intact protein has 2 moles of amino-terminal aspartic acid/mole. Since the light chains have amino-terminal aspartic acid, there are two light chains per mole of protein. The presence of a blocked end group in the heavy chain is consistent with the report of Press *et al.* (1966b), who found an amino-terminal pyrrolidonecarboxylic acid residue in the human γG-immunoglobulin Daw.

In Table V is shown a comparison of the observed amino acid composition of Eu with the composition calculated on the assumption that two heavy chains and two light chains make up the molecule. For most amino acids, the sum differs from the observed composition by less than 5%. The largest difference (10%) occurs in threonine.

There are about 1400 amino acid residues in Eu. In order to determine the amino acid sequence of such a large protein, it is necessary to break down the molecule into smaller fragments in order to simplify the separation of peptides small enough to be sequenced by conventional techniques. Cyanogen bromide cleaves peptide chains specifically at methionine residues (Gross and Witkop, 1962), and for this reason the methionine content of Eu and its chains is important. The heavy chain contains six methionine residues (Table II), and the light chain contains three methionine residues (Table III). Cyanogen bromide cleavage should give 22 fragments. If Eu were composed of two identical light chains and two identical heavy chains, only eleven unique fragments would be observed. The isolation and ordering of these fragments will be described in the succeeding papers (Waxdal *et al.*, 1968a,b).

Limited digestion of γG-immunoglobulin with a number of enzymes produces large fragments analogous to the Fab and Fc fragments resulting from papain digestion. Most of these enzymes have broad specificities. Previous reports have demonstrated that trypsin produces large fragments (Hanson and Johansson, 1960) which resemble those produced by cysteine-activated papain (Schrohenloher, 1963). Tryptic digestion of partially reduced and alkylated Eu under the conditions used in this study appears to be much more rapid than under the conditions described by Schrohenloher (1963), where digestion was still incomplete after 72 hr. Further studies on the partial tryptic digestion of Eu will be reported elsewhere.

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