Heterogeneity of Normal Human Immunoglobulin Light Chains. Nonallelic Variation in the Constant Region of λ Chains*

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ABSTRACT: Immunoglobulin light chains from normal humans were prepared as their mixed disulfides with aminoethanethiol (cysteamine) from the IgG fraction of 31 individual units of plasma. Tryptic digests of the 31 light-chain preparations were characterized by automated peptide analysis in an attempt to locate peptide differences which might reflect genetic polymorphism. The peptide patterns were complex but highly reproducible and no evidence of quantitative or qualitative polymorphism other than that due to the "Inv peptides" was found. Most of the peptides from the C region of κ and λ Bence–Jones proteins were identified in the light-chain digests from normal individuals and a number of these were quantitated. Peptides corresponding to λ chains having serine and glycine at position 153 were identified in all of the light chains examined. This high frequency of finding individuals with two types of chain rules out an explanation in terms of a simple genetic polymorphism. Quantitation of the two peptides in 6 normal light-chain preparations indicated that the relative amounts of the two types of λ chains was in the range of 3 or 4 to 1 for the serine^{153} *vs.* glycine^{153} types. The implications of these findings with regard to the number of genes for the C region of the λ chain are discussed. A number of variable region peptides were identified, including a new subgroup K_{III} sequence, which was present in all individual light chains.

We have started an investigation into the structure of light chains of immunoglobulins from normal human individuals in an attempt to answer several questions which have arisen from structural studies on Bence–Jones proteins and light chains of myeloma proteins. In particular it is important in understanding the origin of antibody variability to know how much (if any) of the sequence variation seen in Bence–Jones proteins is due to genetic differences between the various myeloma donors, and whether the Bence–Jones proteins are in any way nonrepresentative of normal antibody light chains. We have consequently developed and used methods which allow us to characterize portions of both the "variable" and the "constant" regions of normal individual light chains. The methods can be applied to screening samples from a number of persons and so are potentially valuable for detecting inherited variations in both regions.

To date only one polymorphism has been established in human light chains; the replacement of valine by leucine at position 191[1] in those κ chains which have the Inv (1) antigenic specificity (Baglioni *et al.*, 1966; Milstein, 1966a,b). In contrast to the Inv case, where two amino acids at position 191 are seen only in the heterozygote (Terry *et al.*, 1969), amino-terminal sequence heterogeneity up to position 4 has been found in all normal individual light chains so far examined (Hood *et al.*, 1969). Furthermore two peptides (Ala,-Thr,Leu,Ser,Cys,Arg) and (Val,Thr,Ile,Thr,Cys,Arg) corresponding to position 19–24 in the variable region of the κ chain have also been shown to be nonallelic (Milstein *et al.*, 1969; Smithies *et al.*, 1969). In λ chains the peptides (Ser, His, Arg) and (Ser, His, Lys) both corresponding to position 188–190 in the constant region have been identified in 10 successive individual light chains (Ein, 1968). Once again the high frequency of finding two amino acids at one position rules out any explanation in terms of a simple genetic polymorphism.

In this paper we describe the preparation of light chains from single individuals and the characterization of tryptic peptides of these light chains by column chromatography. Amino acid analysis of successive fractions from preparative scale chromatograms led to the identification of most of the C-region peptides of both κ and λ as well as a small number of V-region peptides. Two peptides corresponding to the λ C-region positions 151–157 were found in 31 consecutive individual light chains.

Materials and Methods

Plasma. Whole blood freshly drawn into standard plastic bags containing acid–citrate–dextrose solution was kindly made available to us by the American Red Cross, Madison, Wis. Heparinized blood proved unsuitable. Plasma was prepared by centrifuging twice, the second time for 15 min at 15,000 rpm on a Servall refrigerated centrifuge. The pH was made 6.3 with 1 M citric acid and the plasma was frozen for storage; after thawing it was recentrifuged at high speed to remove precipitated protein. Frozen and thawed plasma was more satisfactory on the column than freshly acidified plasma.

γ-Globulin Preparation. A single-column procedure was devised, based on the method of Levy and Sober (1960), which

* This is paper 1462 from the Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received April 12, 1971.* D. G. was supported by a postdoctoral fellowship from the National Research Council of Canada. Other support was from National Science Foundation GB-4362 and National Institutes of Health GM-15422.

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[1] Amino acid numbering is based on the sequence of protein Ag for the κ chain and on protein Sh for the λ chain. Proteins or peptides having certain residues at a given position are referred to by the amino acid and a superscript, Eg. Proteins or alligned peptides having serine at position 153 are referred to as Ser^{153} proteins or Ser^{153} peptides. κ and λ peptides corresponding to positions *n* to r are referred to as K_{n-r} and L_{n-r}, respectively. V_λ and C_λ refer to the variable and constant part of the λ chain. Other abbreviations used are: TEA, triethylamine; DTT, dithiothreitol; PTH-, phenylthiohydantoin; Cys-*S*-*S*-ethylamine refers to the mixed disulfide of cysteine and cysteamine (aminoethanethiol).

permitted one unit of plasma to be processed each day at the same time as the column was equilibrated for the next run. The column dimensions were 150 × 6.2 cm. The bottom 50 cm was packed with DEAE-cellulose (standard grade "Select-acel," Schleicher & Schuell Co., Keene, N. H.) as its phosphate; the upper 75 cm was packed with Sephadex G-25 coarse beads. Up to 300 ml of acidified plasma was applied to the column. The eluting buffer was pH 6.3 ammonium phosphate, 0.017 M in phosphate. When the back of the plasma zone was about 30 cm down the Sephadex column, approximately 200 ml of concentrated "stripping buffer" was applied to the column. The stripping buffer was pH 5.7 ammonium phosphate, 1.7 M in phosphate. Ammonium salts were used to avoid solubility problems at cold room temperatures and the pH of the stripping buffer (5.7) was chosen so that the column reequilibrated rapidly to pH 6.3 as the 0.017 M eluting buffer followed the stripping buffer. The effluent was ultraviolet monitored and the γ-globulin peak was pooled. The pooled fractions were concentrated overnight to a volume of 10–25 ml using an Amicon 700-ml protein concentrator at 35 psi of N_2 with a UM-2 membrane. The final γ-globulin solution with the washings from the concentrator was adjusted to a volume of 50 ml and the yield was measured by ultraviolet absorption.

Dissociation Intrachain Disulfide Bonds. Up to 2.2 g of γ-globulin in 50 ml of 0.017 M ammonium phosphate buffer was diluted to 100 ml with distilled water. To this was added 10 ml of 4 M Tris buffer (4 M in Tris base, 0.06 M in HCl, and 0.04 M in EDTA as the free acid) and a small crystal of phenol red WS. Solid DTT (77 mg) was added to give a final concentration of 0.005 M. After 5 min the solution was made 0.25 M in cystamine dihydrochloride by adding 10 ml of a centrifuged and filtered 57% w/v solution. This procedure forms a mixed disulfide with the exposed cysteine residues (Smithies, 1965). After a further 5 min 280 mg of solid recrystallized iodoacetamide was added to make the solution 0.015 M. This removes the cysteamine generated by the disulfide interchange reaction and prevents further disulfide interchanges from occurring. Five minutes later 72 g of solid urea was added to give a final concentration of 6 M and the solution was acidified to pH 3.1 by adding 40 ml of 4 M formic acid. Water was added to give a final volume of 200 ml. Separation of light chains from the resulting mixture was carried out using the segmented G-100 column described below. Although the light chains obtained as their *S*-*S*-ethylamino derivatives are free from contamination with heavy chains, the yields are only about 50% of the theoretical. Nevertheless the method of isolation was well suited to our aim of preparing a large number of samples so that the limited yield was accepted. Indications were obtained (see Results) that the light chains prepared in this way are enriched in λ relative to κ.

Separation of Light and Heavy Chains. Columns of Sephadex G-100 (40- to 120-μ bead, 8.5 × 85 cm) equilibrated in the cold room with 5 M urea–0.05 M formic acid (Franěk *et al.*, 1964) were used to separate light and heavy chains. To permit high flow rates which did not change with repeated use, the columns were packed in a single thin-walled (1/8-in. wall) plastic column in three segments each separated by an inner 2.5 cm length of thick-walled plastic cylinder (0.25-in. wall) filled with Sephadex G-25 coarse beads and closed at the bottom by a stainless steel screen. The bottom G-100 segment also had a small length of G-25 below it. The short cylinders which fitted loosely inside the thin-walled long column were held in position by deforming the external cylinder with a circumferential stainless steel hose clamp pressing against three small lengths of 1/8-in. rod 120° apart.

Dissociated light and heavy chains from up to 2.2 g of γ-globulin in 200 ml of solution were successfully processed on this column. The protein in 6 M urea was layered under the 5 M urea on top of the column. The eluting solution of 5 M urea–0.05 M formic acid was filtered through a Millipore microfiber prefilter before use. Flow rates of around 1.5–2 ml/min were usual; the column effluent was ultraviolet monitored. Suitably pooled fractions were concentrated on UM-2 Amicon membranes and recovered in a total volume of 10–12 ml.

Preparation of Fully Reduced Light Chains as S-S-Ethylamine Derivatives. Up to 8 batches were processed at one time. Solid urea (4 g) was added to 10–12 ml of each light-chain solution in 5 M urea–0.05 M formic acid. A freshly made solution (0.1 ml) of DDT (100 mg/ml of water) was added to give 0.005 M and 25% trimethylamine was added dropwise to give a pH of 9–10. After 5 min 1.4 ml of 57% (w/v) cystamine dihydrochloride was added and the pH brought back up to between 9 and 10. Five minutes later 1 ml of freshly made 0.21 M aqueous iodoacetamide was added to give 1.5 equiv of the thiol groups present. After 5 min the light-chain derivatives were precipitated with 50 ml of methanol. None of the reagents and their by-products are precipitated in this step so that the protein can be effectively freed of them by further alcohol washes. The mixture was centrifuged for 10 min at high speed using polypropylene tubes. The precipitate was washed by finely dispersing it in more methanol with a Teflon homogenizer selected to fit the centrifuge tube, and was recentrifuged. Two methanol washes and two ether washes were used. The final ether-washed precipitates and tubes were put into a 37° incubator for about 20 min and then allowed to dry at room temperature with occasional pulverizing with a spatula.

Tryptic Digests. For analytical peptide chromatograms 23 mg of light chain (as the mixed disulfide with cysteamine) was dissolved in 2.3 ml of distilled water in a Potter-Elvehem Teflon homogenizer. Any insoluble material (usually very little) was removed by centrifugation. Duplicate digests were made by taking 1 ml of the light-chain solution and adding to it 0.1 ml of a freshly made solution of Worthington L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (2 mg/ml in 10^{-3} M HCl). After mixing, 0.1 ml of 1 M $NaHCO_3$ was added; a precipitate formed. Digestion was carried out at 37° for 2.5 hr on a rotator which kept the precipitate suspended. The reaction was stopped by adding 0.1 ml of 4 M formic acid. At this stage so little precipitate was present that the digests could be applied directly to the column of the peptide analyzer. For preparative runs the same procedure was used with all the volume scaled up in proportion.

Peptide Analyses. BUFFER SYSTEMS. Analytical and preparative chromatograms were both carried out using a Technicon peptide analyzer with a 6 mm × 35 cm column of finely graded Dowex 50 (Technicon Type P resin) at 50°. Several gradients were tested for their ability to give good separations, including pyridine- and *N*-ethylmorpholine-based systems. The best results were obtained with a slight modification of the sodium citrate buffer system described by Technicon. This system gave a gradient which was nearly linear in pH from 3.1 to 10.8. For most of the preparative runs a triethylamine-based buffer system was used which gave a very similar pattern of peptides but had the added advantage of being volatile (TEA–acetate and TEA–formate could be removed completely under good vacuum). The buffer compositions and gradient construction are outlined in Table I.

DETECTION SYSTEM. For most of the chromatograms the sys-

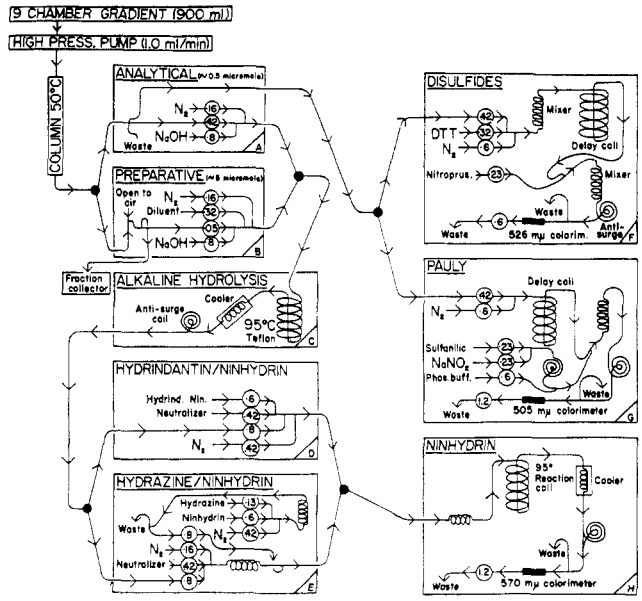

FIGURE 1: (A, B, C) The sodium hydroxide for hydrolysis was 13% w/v NaOH plus 2% ARW-7 (an alkali-resistant wetting agent (Technicon Corp.)). Diluent was 2% ARW-7 in water. (D) Hydrindantin–ninhydrin stock solution contained 1.5 g of hydrindantin, 20 g of ninhydrin, 650 ml of methyl Cellosolve, and 350 ml of 4 N sodium acetate (pH 5.5). The working solution was prepared by mixing 3 volumes of 50% methyl Cellosolve and 1 volume of ninhydrin stock solution. (E) Hydrazine solution was prepared fresh every day or every second day and contained 130 mg of hydrazine sulfate, 250 ml of water, 15 ml of Brij-35 (50% w/v solution), 75 ml of 4 N sodium acetate (pH 5.5), made up to 500 ml with methyl Cellosolve. Neutralizer contained the following: 360 ml of 4 N sodium acetate, 1120 ml of glacial acetic acid, and 40 ml of Brij-35 (50% w/v) solution) made up to 4 l. with water. Air-stable ninhydrin working solution was prepared in 8-l. batches; it contained 80 g of ninhydrin, 4 l. of methyl Cellosolve, 1600 ml of 4 N sodium acetate (pH 5.5), 400 ml of glacial acetic acid, and was made up to volume (8 l.) with water. (F) Nitroprusside solution consisted of 1% w/v sodium nitroprusside in water plus 1% Brij-35 (50% w/v solution). DTT stock solution contained 2 lb of K_2HPO4, 1 lb of KCl, 120 g of KOH, 40 ml of ARW-7, made up to 1 gal. with water; the working solution consisted of 23 mg of DTT, and 50 mg of sodium cyanide dissolved in 500 ml of the stock phosphate buffer. (G) Phosphate buffer consisted of the DTT stock solution (see F) diluted with an equal volume of water. Sulfanilic acid reagent was 0.05 M sulfanilic acid dissolved in 0.35 M HCl. Sodium nitrite solution was 0.2 M sodium nitrite in water. The circled numbers refer to nominal pump tube capacities in milliliters per minute.

TABLE 1: Composition of Buffer Gradients.

Gradient Chamber	Buffer Systems: Sodium Buffers[a]	Triethylamine Buffers[b]
1	Buffer 1	Buffer 1
2	Buffer 1	Buffer 1 + buffer 2, 2:1
3	Buffer 1 + buffer 2, 2:1	Buffer 1 + buffer 2, 1:5
4	Buffer 1 + buffer 2, 1:5	Buffer 2
5	Buffer 2	Buffer 3
6	Buffer 2	Buffer 3
7	Buffer 2 + buffer 3, 1:1	Buffer 4
8	Buffer 3	Buffer 4
9	Buffer 3	Buffer 5

[a] Buffer 1: 0.05 M trisodium citrate–0.05 M NaCl, pH 3.1 with HCl; buffer 2: 0.05 M trisodium citrate–0.05 M NaCl, pH 5.1 with HCl; buffer 3: 0.05 M trisodium citrate–0.05 M NaCl, pH 10.8 with solid Na_2CO_3. [b] Triethylamine was refluxed for 2 hr with an excess of ninhydrin, then distilled at 1 atm before use. Buffer 1: 0.05 M TEA, pH 3.1 with formic acid; buffer 2: 0.1 M TEA, pH 5.1 with acetic acid; buffer 3: 0.1 M TEA, pH 6.1 with acetic acid; buffer 4: 0.1 M TEA, pH 7.0 with acetic acid; buffer 5: 0.1 M acetic acid, pH 10.8 with TEA.

tem was set up to determine ninhydrin color after alkaline hydrolysis rather that direct ninhydrin color. In the early stages of the work the conventional ninhydrin–hydrinantin system was used but this was later replaced by the more convenient air-stable ninhydrin system employing separate ninhydrin and hydrazine solutions. In most of the analytical work the second channel of the peptide analyzer was set up to detect disulfide-containing peptides using a modification of the system described by Barber (1966). On occasion the Pauly reaction was used to detect histidine- and tyrosine-containing peptides. The flow diagram and reagents are shown in Figure 1.

Desalting of Peptides. In preparative peptide chromatograms using the sodium citrate buffer system, it was necessary to desalt the fractions before they could be used for amino acid analysis or other determinations. It was rarely found advisable to pool fractions; the following procedure is that used for desalting individual fractions. The fraction, containing about 5 ml of peptide solution in sodium citrate buffer, was acidified by the addition of 2 ml of 0.5 N HCl. It was then applied to a column containing *ca.* 2 ml of Dowex 50-X4, 250–300 mesh in the H^+ form, equilibrated with 0.1 M formic acid. After application of the sample, the column was washed twice with 5 ml of 0.1 M formic acid. The peptide material remained absorbed to the resin during this washing. The peptides were then eluted from the column with 5 ml of 0.5 M triethylamine and evaporated to dryness.

Edman Degradation. A manual method (Niall *et al.*, 1969) was employed, using 1.0 M *N,N'*-dimethylallylamine–trifluoroacetic acid buffer (pH 9.5) in 60% v/v aqueous pyridine. After the cleavage the trifluoroacetic acid was lyophilized and the residue dissolved in 0.5 ml of 0.01 N HCl. The thiazalinone derivatives were extracted with ether. PTH-amino acids were identified by hydrolysis and amino acid analysis (D. Gibson, in preparation).

Hydrazinolysis. Peptide was dissolved in 0.5 ml of 95% hydrazine and sealed under vacuum. Hydrazinolysis was carried out at 80° for 12 hr. The C-terminal amino acid was identified by direct amino acid analysis.

Amino Acid Analysis. Amino acid analyses were made with the Technicon TSM-1 amino acid analyzer. Hydrolysis of multiple fractions was conducted in an evacuated desiccator at 105°. It was found advantageous to add several drops of constant-boiling HCl to each of the tubes in addition to that added to the desiccator on the outside of the tubes. Hydrolysis was routinely carried out for 24–40 hr.

In the latter part of this work, a single-column 90-min analysis was used exclusively. Based on the "1-hr" TSM-1 system (Ertingshausen *et al.*, 1969) but with an additional buffer (0.3 M NaCl, 0.07 N NaOH, solid boric acid to pH 9.4, and 1% Brij 35) to bring the basic amino acids off the long column, the system gave an effective increase in sensitivity of 2X due to the use of the entire sample for analysis and in addition gave

improved lysine–histidine separation and improved arginine sensitivity. Contaminants were removed from the earlier buffers by online filters containing *ca.* 500 cm^3 of Fisher Rexyn 101 (sulfonic acid resin), 40–100 mesh in the Na^+ form.

Results

Peptide Maps of 31 Normal Individual Light Chains. Light chains as their Cys-*S*-*S*-ethylamine derivatives from 31 individual sera were digested with trypsin and subjected to analytical scale peptide analysis. With the exception of three samples (one each from a Mexican, a North American Indian, and a Negro), the light chains were from unselected donor sera obtained from the Red Cross. All samples were subjected to duplicate analyses using independently prepared tryptic digests. Two-channel tryptic peptide maps of four different individual light chains are shown in Figure 2. This figure illustrates both the high degree of reproducibility of the method and also the clear difference involving the "Inv"peptides. As has been reported previously (Terry *et al.*, 1969), light chains of Inv type (1,3) showed an additional peptide compared to the light chains of Inv type (−1,3). The extra peptide was *S*-*S* positive (Figure 2) and its composition (Leu,-Tyr,Ala,Cys-*S*-*S*-ethylamine) as determined by amino acid analysis of the relevant fractions from preparative scale maps corresponded to the expected "Inv 1" peptide. A total of 6 out of 31 light chains were found to be positive for the extra peptide, giving a frequency close to that expected for the Inv (1,3) heterozygote (Ritter *et al.*, 1964).

Peptide chromatograms could be compared in detail by superimposing two charts over a light box. Over the course of weeks minor changes in the exact elution position of certain groups of peptides occurred, but these could be attributed to variations in the elution buffers. To help distinguish between true peptide differences and those due to changes in the conditions, a single individual light chain was run periodically as a "standard". Careful comparisons of all of the maps failed to reveal any evidence of polymorphism other than that due to the Inv peptides. Differences between the maps in all cases appeared to be due to minor quantitative variations in certain peptides and it was impossible to divide the light chains on this basis into any distinct types.

In order to identify as many peptides as possible without extensive further purification, a novel approach was undertaken. The method involved separate amino acid analysis of each fraction from a preparative scale chromatogram. In effect this generates 16 amino acid specific chromatographic traces which can be inspected for congruencies from which peptide compositions can be deduced. This approach has been used only to a limited extent in the past mainly due to lack of amino acid analyzer time, but with the use of a fully automated high-speed analyzer (Ertingshausen *et al.*, 1969) this practical problem has been largely overcome. Analysis of successive fractions in this fashion has proved extremely useful and we have used it extensively throughout this study. Its value lies in its ability to permit identification of overlapping peptides and minor component peptides without the necessity of extensive peptide fractionation. In addition it provides a means of determining the relative yields of peptides without the introduction of errors due to imprecise pooling of fractions.

Preparative scale (100 mg) tryptic peptide chromatograms of two individual light chains, Inv types (−1,3) and (1,3), were employed for detailed comparison by amino acid analysis of successive fractions. Figure 3 shows the preparative chromatogram obtained with the Inv (−1,3) light chains. After desalting

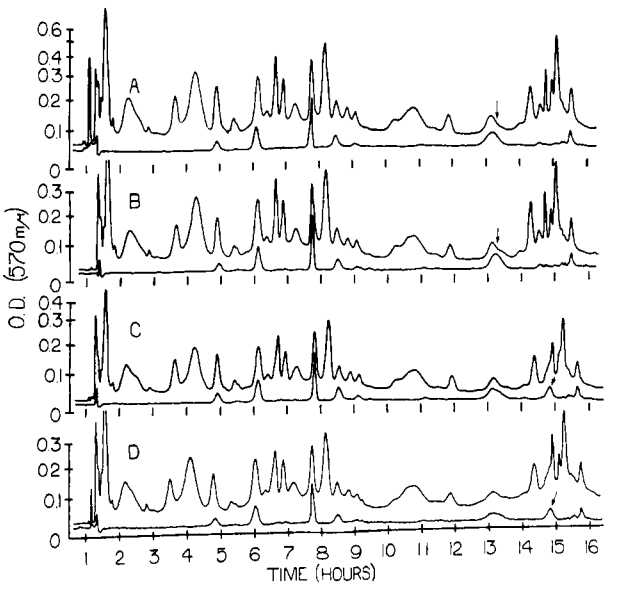

FIGURE 2: Analytical scale tryptic peptide chromatograms of the light chains from 4 different individuals run on Technicon "type P" resin with sodium citrate buffers (Table I). The upper trace in each chromatogram represents the ninhydrin-positive material after alkaline hydrolysis, while the lower trace gives the *S*-*S*-positive material (see Figure 1F). Since all cysteines were in the *S*-*S*-ethylamine form, the lower traces are specific for cysteine-containing peptides. Each sample contained 20 mg of digested light chain. Light chains A and B were Inv (−1.3) while C and D were Inv (1,3). Arrows indicate the position of the "Inv 3" peptide (Val,-Tyr,Ala,Cys-*S*-*S*-ethylamine) in the upper two chromatograms and the additional "Inv 1" peptide (Leu,Tyr,Ala,Cys-*S*-*S*-ethylamine) in the lower two chromatograms.

of the 180 individual fractions an aliquot of each was hydrolyzed and subjected to amino acid analysis. Although a high "background" level of amino acids was typical of the chromatograms of normal light-chain digests, we have found it to be entirely absent from peptide chromatograms of homogeneous proteins such as Bence–Jones proteins or the chains of hemoglobin. We presume it represents an almost continuous distribution of partially resolved low-yield peptides from the variable region of normal light chains.

Constant Region Peptides. Twenty-five of the expected thirty-four tryptic peptides of the constant region of κ and λ Bence–Jones proteins were identified in the two chromatograms (partial cleavage at Cys-*S*-*S*-ethylamine was expected). Their locations are shown in Figure 3 and in Table II. Table II also includes estimates of the yields of a number of the peptides. Yield determinations were not attempted in all cases because of the high "background" level of amino acid recoveries in many areas of the chromatograms. Where yields were determined, they were made by summing the recoveries of individual amino acid constituents of a peptide over all fractions comprising the peak after drawing in an appropriate "base line" for that amino acid: a procedure which tends to give a conservative estimate. On the basis of the weighed amount of protein in the digest, the average yields for the κ C-region peptides were 39 and 33%, respectively, in the two individual light chains and for the λ peptides the yields were 16.6 and 16.3%. Both of these average recoveries are low compared to the reported recoveries of the C-terminal peptides of normal light chains (40–53% for the κ C-terminal peptide and 16–24% for the λ C-terminal peptide, Milstein, 1965) but since we made no attempt to dry the samples to

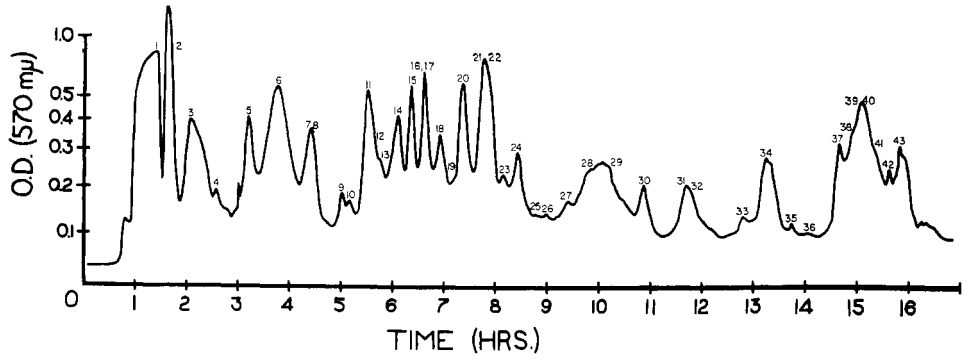

FIGURE 3: Preparative scale tryptic peptide chromatogram of a normal individual light chain (Inv −1,3). Light chain (100 mg) was digested with trypsin and chromatographed on "type P" resin using the sodium citrate buffer system (Table I). Approximately 5% of the effluent was used for the continuous analysis of total peptide (ninhydrin after alkaline hydrolysis), the remainder being collected as 5-min fractions. For identity of some of the numbered peaks, refer to tables II and VI.

constant weight, the difference is probably due in part to a higher hydration of our preparations.

As expected, the two "Inv" peptides $K_{191-194}$ (Val,Tyr,Ala,-Cys-*S*-*S*-ethylamine) and (Leu,Tyr,Ala,Cys-*S*-*S*-ethylamine) were recovered in yields approaching half-molar relative to the other constant region peptides in the Inv (1,3) light-chain digest (Terry *et al.*, 1969). Partial tryptic cleavage of the peptide bond on the carboxy side of the Cys-*S*-*S*-ethylamine at K_{194} explains the recovery of a slight but significant amount of the peptide(s) $K_{191-207}$ as well as the lower than expected yields of the peptides $K_{191-194}$ and $K_{195-207}$. The rate of hydrolysis by trypsin of the peptide bond on the carboxy side of Cys-*S*-*S*-ethylamine varied considerably for the different cysteines and presumably is affected by the amino acids in neighboring positions. For example, the Cys-*S*-*S*-ethylamine peptide bond at position K_{134} was completely hydrolyzed, as reflected in the molar yields of the peptides $K_{127-134}$ and $K_{135-142}$ while the bond between the K_{23} Cys-*S*-*S*-ethylamine and K_{24} arginine was not hydrolyzed at all. We have found the Cys-*S*-*S*-ethylamine derivatives to be of considerable value in conjunction with trypsin, since unlike the derivatives prepared by S aminoethylation, the basic side chain on the cysteines can be removed at will.

TABLE II: Position and Yield of Constant Region Peptides from Normal Light Chains.

Peptide	Peak No. (Figure 3)	Yield (% of Weighed Protein)	
		Light Chain 018 (Inv −1,3)	Light Chain 932 (Inv 1,3)
K_{108}	18		
$K_{127-134}$	11	42.9	30.5
$K_{135-142}$	38	41.7	36.2
$K_{143-145}$	15	41.6	32.6
$K_{146-149}$	40		
$K_{150-169}$	2		
$K_{184-188}$	16		
$K_{189-190}$	40		
$K_{191-194}$(Val^{191})	32	34.8	14.9
$K_{191-194}$(Leu^{191})	34–35	Absent[a]	10.9
$K_{191-207}$(Val^{191})	37		
$K_{191-207}$(Leu^{191})	37		
$K_{195-207}$	21		19.9
$K_{208-211}$	34	35.0	28.6
$K_{212-214}$	20		
$\lambda_{112-130}$	3		
$\lambda_{131-135}$	22		
$\lambda_{151-157}$(Ser^{153})	8	12.0	12.7
$\lambda_{151-157}$(Gly^{153})	9	3.5	4.9
$\lambda_{153-167}$	5	18.3	16.9
$\lambda_{168-172}$	14	16.4	15.5
$\lambda_{173-187}$	28		
$\lambda_{188-190}$(Arg^{190})	41		
$\lambda_{191-194}$	31		
$\lambda_{195-205}$	21		
$\lambda_{206-213}$	7	15.1	16.5

[a] Owing to high background, the presence of as much as 2% of this peptide might have gone undetected.

The λ constant region peptide $L_{151-157}$ (Ala,Asp,Ser_2,Pro,-Val,Lys) was recovered in considerably lower yield relative to the other λ constant region peptides in the light chains from both individuals. This low recovery was explained by the subsequent identification of a minor peptide corresponding to this same region of the λ chain in the two chromatograms. The primary data which permit the identification of the peptides are presented in Figure 4. This figure shows the amino acid analyses of successive fractions from a small part of the large-scale chromatogram illustrated in Figure 3. The figure also serves to demonstrate the potentialities of the method of amino acid analysis of successive fractions.

Inspection of the plots of each amino acid in Figure 4 shows that two partially resolved peptides are present in fractions 35–40. Peptide 7 which peaks in fraction 37 was known to be cysteine positive from the analytical chromatograms (Figure 2). It contained no arginine or lysine, and corresponded in composition to the λ C-terminal peptide $L_{206-213}$ (Thr_2,Ser,-Glu,Pro,Ala,Val,Cys-*S*-*S*-ethylamine). Peak 8, occurring in fraction 38 corresponds in composition to the lysine-containing peptide $L_{151-157}$ (Asp,Ser_2,Pro,Ala,Val,Lys). These two peptides are readily distinguished and identified by the procedure of analyzing all fractions even though they peak in adjacent fractions. Their yields were determined on the basis of their unique residues: threonine and glutamic acid for the former peptide, aspartic acid and lysine for the latter. The composition of peak 9 in the chromatogram corresponds to (Asp,Ser,-

TABLE III: Composition of λ C-Region Peptides Isolated from the Light Chains of a Single Individual.

	$L_{151-157}$ (Ser)	$L_{151-157}$ (Gly)	$L_{206-213}$	$L_{136-150}$	$L_{136-150}$ (Mz)[a]
Cys			+[b]		
Asp	1.0	1.0		1.0	1
Thr			1.9	1.0	1
Ser	1.9	1.0	1.1	1.0	1
Glu		0.3	1.1		
Pro	1.2	0.8	0.9	0.8	1
Gly		1.0		1.1	1
Ala	1.1	0.9	1.0	2.1	1
Val	0.9	0.9	1.0	2.0	3
Met					
Ile				1.0	1
Leu				0.8	1
Tyr				0.9	1
Phe				1.2	1
His					
Lys	0.8	0.8		1.0	1
Arg		0.3			
Trp				nd[c]	1
Peptide system	TEA	TEA	TEA	TEA	

[a] Composition taken from the sequence of the variant λ Bence-Jones protein Mz (Milstein *et al.*, 1967). [b] On acid hydrolysis Cys-*S*-*S*-ethylamine-containing peptides give rise to a significant amount of cystine. Cystine was present in the hydrolysate of this peptide but it was not quantitated. [c] nd = not determined.

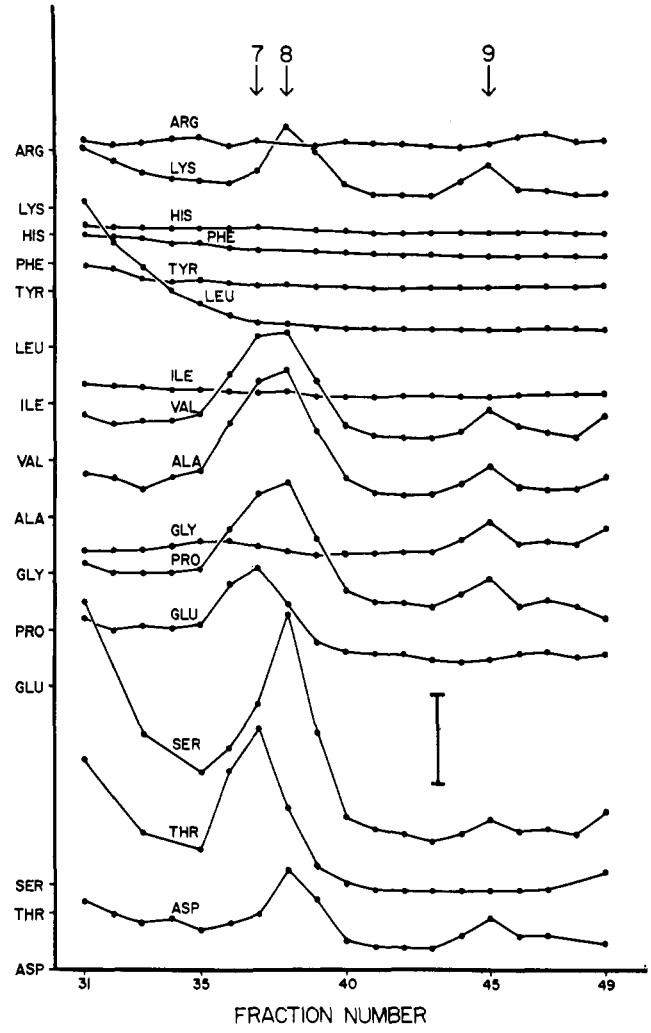

FIGURE 4: Amino acid analysis of the successive fractions (31–49) from a preparative scale tryptic peptide chromatogram. After adding a fixed amount of norleucine to each fraction, an aliquot of each was hydrolyzed and subjected to amino acid analysis. The recoveries of each residue were then plotted, using separate base lines for clarity. Inspection of the composite graph enables the identification of 3 peptides, 7, 8, and 9, peaking in fractions 37, 38, and 45, respectively. The vertical bar represents 50 nmoles of amino acid in the analysis. Since 0.1 of the fraction was used for analysis, it corresponds to 500 nmoles in the total fraction.

Pro,Gly,Ala,Val,Lys) which differs from peak 8 by a single residue (Gly for Ser). In later work using the triethylamine buffer system, the same three peptides were obtained in more homogeneous form on single chromatograms and their compositions are given in Table III. Edman degradation established the partial sequences Ala·Asp·Ser·(Ser·Pro·Val·Lys) and Ala·Asp·Gly·(Ser·Pro·Val·Lys) for the peptides 8 and 9, respectively. Incubation of the peptides with a mixture of carboxypeptidases A and B (Ambler, 1967) liberated only lysine in both cases suggesting that proline was adjacent to or two residues removed from the C terminus, thus blocking the action of carboxypeptidase A. Incubation with carboxypeptidase B followed by hydrazinolysis and direct amino acid analysis established the C-terminal sequence of both peptides as Val·Lys. This in conjunction with the negative result using carboxypeptidase A indicates that the most probable sequences are Ala·Asp·Ser·Ser·Pro·Val·Lys and Ala·Asp·Gly·Ser·Pro·Val·Lys. Both of these sequences have been found in λ Bence-Jones proteins at position 151–157 but the latter has only been seen twice out of a total of 24 proteins. The Gly^{153} sequence was initially reported in the Bence-Jones protein Kern (Ponstingl *et al.*, 1968) and since then has been found in one additional λ protein, BJP-119 (Hess and Hilschmann, 1970). The latter authors suggested that the low frequency of occurrence of Kern-type Bence-Jones proteins might be the result of germ-line polymorphism.

Our results however demonstrate that Kern-type (Gly^{153}) λ chains are present as a minor component in all normal individual light chains. Peak 9, corresponding to the Kern-type peptide was clearly present in the analytical scale chromatograms of all 31 of the individual light chains examined. The major $L_{151-157}$ peptide (Ser^{153}) cochromatographed with the λ C-terminal peptide ($L_{206-213}$) and consequently could not be seen as a discrete peak on the analytical scale maps. Nevertheless its presence in all cases could be inferred from the size of the combined peak (7 + 8) in Figure 3 relative to peak 9. Thus in no case did peak 9 become equal in size to peak (7 + 8) as would have occurred if all of the λ chains possessed glycine at position 153. These findings mean that all 31 individuals possessed some λ chains with serine and some with glycine at position 153.

The relative amounts of the peptides $L_{151-157}$ (Ser^{153}) and $L_{151-157}$ (Gly^{153}) and the λ C-terminal octapeptide were determined in light chains from four additional individuals and these yields together with the original two are given in Table IV (two of the determinations were from chromatograms developed with the triethylamine buffer system which gave

TABLE IV: Yields of $L_{151-157}$ Peptides in Normal Individual Light Chains.

Individual Light Chain	Yield[b] of $L_{151-157}$ Peptides		Ratio of Ser/Gly at Position 153
	Ser^{153}	Gly^{153}	
703[a]	77	19	4.1
956[a]	58	29	2.0
018	80	23	3.4
932	77	30	2.6
Clark	78	25	3.1
Otis	83	19	4.4

[a] Determinations made using triethylamine buffer system for chromatography. The tryptic digests in these two cases had been first fractionated on Sephadex G-25 before Dowex 50 chromatography. [b] Yields are expressed as per cent of the recovery of the λ C-terminal peptide $L_{206-213}$ on the same chromatogram.

TABLE V: Variation in the C Region of Bence-Jones Proteins.

Position 122	Position 191	No. of Proteins	Ref
	A. κ		
Asp	Val	2	*a*
Asx	Val	2	*b*
	Val	5	*c*
Asp	*LEU*[t]	3	*d*
Asx	*LEU*	1	*e*
	LEU	1	*f*
ASN	Val	1	*g*

Position 144	Position 153	Position 172	Position 190	No. of Proteins	Ref
		B. λ			
Ala	Ser	Lys	Arg	6	*h*
	Ser	Lys	Arg	1	*i*
	Ser		Arg	1	*j*
Ala		Lys		6	*k*
Ala				3	*l*
	Ser			8	*m*
Ala	*GLY*	Lys	Arg	1	*n*
	GLY			1	*o*
Ala	Ser	Lys	*LYS*	2	*p*
	Ser		*LYS*	3	*q*
			LYS	1	*r*
VAL	Ser	*ASN*	Arg	1	*s*

[a] Ti: Suter *et al.* (1969); Rad: Milstein (1966a,b). [b] Eu: Cunningham *et al.* (1968); Mil (BJ3): Dreyer *et al.* (1967). [c] B6: Milstein (1969); HBJ4, Ste, Hac: Hood and Talmage (1970); Man: Milstein (1970). [d] Roy: Hilschmann (1967), Ker, BJ: Milstein (1966b). [e] Dil: A. B. Edmundson (personal communication). [f] Fr4: Milstein (1969). [g] Ag: Titani *et al.* (1969). [h] X: Milstein *et al.* (1968); New: Langer *et al.* (1968); Ha, Bo: Putnam *et al.* (1967); Sh: Wikler *et al.* (1967); Vil: Ponstingl and Hilschmann (1969). [i] Hul: Edmundson *et al.* (1968). [j] Bau: Hess and Hilschmann (1970). [k] Wil, Bu, We, Li, Lw, Lb: Milstein *et al.* (1967). [l] Fr, Sch, Mil: Milstein *et al.* (1967). [m] Be, Fr, Ku, St, Nei, Pf, Pro, Yo: Hess and Hilschmann (1970). [n] Kern: Ponstingl *et al.* (1968). [o] BJP-119: Hess and Hilschmann (1970). [p] HS5, HS92: Hood and Ein (1968). [q] Nev: Edmundson *et al.* (1968); BJP-111, Vor: Hess and Hilschmann (1970). [r] Vin: R. M. Pink (quoted by Hess and Hilschmann, 1970). [s] Mz: Milstein *et al.* (1967). [t] Unusual Residues are in capitals and italicized.

complete resolution of the 3 peptides). Although on first inspection there appears to be some variation in the ratio of the two peptides in different individuals, consideration of the yields rather than the ratios shows that these differences are not significant. Omitting the data of light chain 956, in which the low yield of the Ser^{153} peptide could have resulted from differential loss on the preliminary gel filtration step in which an unusually narrow cut was used, the average yields of the Ser^{153} and Gly^{153} peptides were 79.0 ± 2.6% and 23.2 ± 4.5% in 5 individual light chains. The average sum of the two peptides was 102.2 ± 4%. This indicates that all of the λ chains can be accounted for by the two sequences at these positions in the relative proportions of 3–4:1.

Other Constant Region Variants. Amino acid substitutions have been reported at 2 positions in the C region of κ Bence-Jones proteins and at 4 positions in the C region of λ Bence-Jones proteins. All of the reported substitutions are summarized in Table V. In view of the fact that no inherited differences are known for the λ chains, it would be of considerable value to know the frequency of occurrence of the valine-alanine substitution at L_{144} or the asparagine-lysine substitution at position L_{172} in normal individual light chains. We recovered the λ constant region tryptic peptide $L_{126-150}$ (Ala^{144}) as a homogeneous peak (composition Table III) from two individual light chains when digests were chromatographed on Dowex 50 after preliminary Sephadex G-25 fractionation. We did not locate the Val^{144} peptide in the two chromatograms but we cannot rule out the possibility that it was present but missed.

Variable Region Peptides. In spite of the high background level of peptides throughout the chromatogram, 10 variable region peptides were clearly identified.

The position of elution of these peptides in the chromatogram is shown in Table VI along with their yields relative to κ or λ constant region peptides. Peptides marked with a small *c* were discrete peaks on the analytical peptide chromatograms. Careful inspection of the analytical chromatograms of the 31 individual light chains showed that in no cases were any of these peaks absent. Their relatively high yields suggest that the corresponding sequences are common to a large proportion of the light chains.

Peak 43 (Figure 3) corresponds to a mixture of the two major peptides from the κ chain near the first cysteine residue (Ala,Thr,Leu,Ser,Cys,Arg) and (Val,Thr_2,Ile,Cys,Arg). The very basic nature of these peptides, resulting from the failure of trypsin to cleave the Cys-*S*-*S*-ethylamine–arginyl bond, provided the basis for an effective method for their isolation and an analysis of their heterogeneity. The results of this analysis have already been reported (Smithies *et al.*, 1969).

Discussion

It was initially hoped that the quantitative chromatographic system described here would permit the detection of peptides present at the 5% level in digests of normal light chains but

the results have indicated that this is only possible in those sections of the chromatogram that are not occupied by the high yield κ or λ constant region peptides. However inspite of the complexity of the peptide pattern, almost all of the constant region peptides of both κ and λ light chains were identified in normal individuals by using the method of successive amino acid analysis over consecutive fractions. With the exception of the Inv peptides which were recovered in approximately half-molar yields in the Inv (1,3) light chain, all the evidence obtained suggests that the κ C-region peptides are present in essentially molar amounts. This supports the conclusion drawn from Bence–Jones protein structures that the C-terminal half of normal κ chains possess a single amino acid sequence (Putman, 1962; Hilschmann and Craig, 1965). So far as we are aware, this is the first time that this has been established in normal light chains for virtually all of the κ constant region peptides.

Of the four λ C-region peptides which were quantitated, three had yields compatible with a single amino acid sequence for the corresponding part of the λ chain, but in one case ($L_{151-157}$) the yield was lower than expected. The recovery of a second peptide corresponding to this same λ constant region position 151–157 indicated that this region can have either of two sequences determined by a glycine–serine difference at L_{153}. Both of the $L_{151-157}$ peptides were present in all 31 individual light chains analyzed by the mapping procedure. This frequency of occurrence excludes a simple genetic polymorphism as the explanation of the presence of the two sequences. These results are similar to those of Ein (1968) for the Oz peptides ($L_{188-190}$) which indicated that all individuals have λ chains with lysine and arginine at position 190. Unfortunately the chromatographic system used here did not permit us to observe both of the Oz peptides in the normal light chains examined.

Our data and those of Ein from normal individuals do not permit unambiguous assignment of the linkage relationships between the Ser–Gly alternatives at L_{153} and the Lys–Arg alternatives at position 190, but 3 of the 4 possible combinations have been reported in Bence–Jones proteins (Table V). The most common pair seen in Bence–Jones proteins is the Ser^{153}-Arg^{190} type, while less common are the Ser^{153}-Lys^{190} and Gly^{153}-Arg^{190} types. So far no Gly^{153}-Lys^{190} proteins have been found. The nonallelic behavior of the two residues at position 153 and of the two at position 190 together with the observation of 3 linkage combinations in Bence–Jones proteins suggest that there may be more than two genes per haploid chromosome set coding for the C-terminal half of the λ chain.

The quantitative results revealed no significant variation in the relative amount of the $serine^{153}$ and $glycine^{153}$ λ chains in different individuals. The average values for 5 individuals gave a ratio of the serine to glycine peptides of 3–4 to 1. If association between different V and C genes is random one would expect that the relative numbers of the different types of C_λ sequences in normal light chains would directly reflect the relative numbers of the different C_λ genes. In this case the minimum number of genes coding for λ at 153 would be 4 or 5. On the other hand, Hess and Hilschmann (1970) have pointed out that both of the Bence–Jones proteins (Kern and BJP-119) having glycine at position 153 belonged to the minor V_λ subclass having N-terminal tyrosine so that λ having glycine at position 153 may not be associated with all possible V_λ sequences.

Relatively few peptides from the variable region of κ or λ light chains stood out as discrete peaks on the total peptide pattern. Those which did were identified in the analytical scale peptide maps of all 31 individual light chains. All but one of the variable region peptides identified corresponded to 10% or more of the κ or λ chains on the basis of the yield of constant region peptides and in many cases the recovered peptides accounted for more than 25% of the chains. One peptide (peak 10: Glx,Ser,Gly,Val,Pro,Asx,Arg), appears to be homologous to reported sequences at position 55–61 in the κ chain but it represents a new sequence at this position. It is like the linkage group III, sequence Mil (HBJ3), Ala·Ser·Gly·Val·Pro·Asn·Arg· (Dreyer *et al.*, 1967) but with an Ala to Glx substitution. Its presence at about the same level in all 31 light chains as judged by the presence of peak 10 suggests that it is coded for in the germ line of all of these individuals and that it is not a somatic variant.

All the data we have accumulated attest to the remarkable qualitative and even quantitative similarity of both the variable and constant region peptides in different individuals. The only germ-line variation we have seen is the well-documented Leu–Val interchange in the κ constant region at position 191. If there are somatic variations and we have no reason to doubt this, it is possible that they are so numerous that "mutant" peptides unique to specific individuals occur at levels too low for our detection methods. But it is also possible that somatic mutants are so infrequent or the related antibodies are expressed so rarely that they were not detected in any of the 31 individuals tested. We have no unambiguous way of distinguishing between these possibilities at present.

TABLE VI: V-Region Peptides Identified in Normal Light-Chain Digests.

Sequence Position	Composition	Peak No. (Figure 3)	Yield (% of C Region)[d] Individual 018	Individual 932
κ[a]				
κ_{19-24}	$Ala_1Thr_1Leu_1Ser_1Cys_1Arg_1$	43	27	30
κ_{19-24}	$Val_1Thr_2Ile_1Cys_1Arg_1$	43	35	26
κ_{43-45} + λ_{41-43}	$Ala_1Pro_1Lys_1$	24[c]	42	35
κ_{55-61}	$Ala_1Thr_1Gly_1Ile_1Pro_1Asp_1Arg_1$	16	16	21
κ_{55-61}	$Glu_1Ser_1Gly_1Val_1Pro_1Asp_1Arg_1$	10[c]	5	10
κ_{97-103}	$Thr_2Gly_2Gly_1Phe_1Lys_1$	27[c]	15	13
$\kappa_{104-107}$	$Leu_1Glu_1Ile_1Lys_1$	23[c]	28	28
$\kappa_{104-107}$	$Val_1Glu_1Ile_1Lys_1$	20	31	25
λ[b]				
λ_{60-64}	$Ser_2Gly_1Phe_1Lys_1$	30[c]	68	85
$\lambda_{103-111}$	$Leu_2Thr_1Val_1Gly_1Glu_1Pro_1Lys_1$	16	20	28

[a] Ag numbering. [b] Sh numbering. [c] Peptides forming discrete peaks on the analytical scale chromatograms. [d] Yields as a per cent of the mean yield of "molar" C-region peptides on toe same chromatogram.

Acknowledgments

We thank Dr. George P. Smith for many stimulating discussions and for Table V adapted from his Ph.D thesis (Harvard University, 1970). We also thank Dr. Arthur G. Stein-

berg, Case-Western University, Cleveland, Ohio, for Inv typing our samples, Dr. Jack Gary, Interstate Blood Bank, Memphis, Tenn., for sending several units of plasma, and the Badger Regional Blood Center, American National Red Cross, Madison, Wis., for making plasma available to us.

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