Amino Acid Sequence of Rabbit Pneumococcal Antibody I. Light-Chain Cysteine-Containing Peptides†

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ABSTRACT: Rabbit light chains of the b_4 allotype from anti-pneumococcal antibodies of restricted heterogeneity and non-immune immunoglobulins were subjected to tryptic, cyanogen bromide, and limited acid hydrolysis. The resultant peptides were sequenced using either the manual or automated Edman degradation. Sequences were aligned either by overlap or by homology with human light-chain data. Rabbit κ_B light chains have three intrachain disulfide bonds. Three half-cystines were located at positions 23, 134, and 194, homologous with the positions of half-cystines in human light chains, which possess only two intrachain disulfide bonds (23–88 and 134–194). Frangione and Lamm (1970, *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 339) isolated a peptide from rabbit light chains containing half-cystine-88 by homology with human light chains. We located the two additional half-cystines at position 171 in the constant region and in the variable region between positions 40 and 85. This confirms the presence of a disulfide bond between the constant and variable domains which we had previously postulated. A Val–Leu interchange at position 174 indicates that there are at least two b_4 allotype constant-region sequences.

The amino acid sequence of immunoglobulins[1] has in large part been obtained from study of paraprotein tumor products, most of which have no defined antibody activity. Recently, the availability of homogeneous antibodies (Krause, 1970; Haber, 1970) has allowed a comprehensive attack on the primary structure of antibodies elicited through conventional immunization (Hood *et al.*, 1970; Jaton *et al.*, 1970, 1971; Appella *et al.*, 1971; Fleischman, 1971).

In this paper, anti-pneumococcal antibody light chains of restricted heterogeneity and nonimmune heterogeneous light chains are examined with respect to the amino acid sequence of their cysteine-containing tryptic peptides and other larger fragments obtained by cyanogen bromide cleavage and dilute acid hydrolysis. These peptides are located and ordered by homology with the sequence of Bence-Jones protein Ag of the κ I sublcass (Putnam *et al.*, 1966).

Small tryptic peptides were sequenced completely using the manual Edman technique; larger ones of approximately 30 amino acids were sequenced for 17–28 steps using this procedure. In some cases mixtures of two peptides remained after several chromatographic steps. The amounts precluded further attempts at separation and therefore these mixtures were sequenced using strategies suggested by Gray (1968) and successfully applied by Niall and Potts (1970). Unambiguous sequences were deduced in most cases and verified by comparison with those obtained from corresponding homogeneous peptides. Sequences not established in this way must be regarded as tentative. However, homologies with Bence-Jones protein Ag support our assignments. Automated Edman degradation of the larger fragments obtained after cyanogen bromide cleavage and dilute acid hydrolysis enabled us to order several peptides on the basis of overlapping sequences.

The data allow for the tentative placement of an intrachain disulfide bond connecting the constant and variable regions of the polypeptide chain. They also indicate that the gene coding for the constant region of allotype b_4 κ chains in the rabbit may exist in different forms.

Material and Methods

Immunization Procedures. New Zealand white rabbits of b_4b_4 L chain allotype were immunized with either type VIII pneumococcal vaccine (rabbits 2377 and 2388) or with type III pneumococcal vaccine (rabbits K17 and K7) prepared as previously described (Pincus *et al.*, 1970a). Three injections per week of 1 ml of vaccine, containing 5×10^9 cells were given for 4 weeks followed by weekly injections for several months (Kimball *et al.*, 1971). Sera were monitored weekly by electrophoresis on cellulose acetate (Beckman Microzone) and selected for electrophoretic restriction in the γ-globulin region (Pincus *et al.*, 1970b). If a restricted pattern was observed to remain constant over several successive bleedings, the sera were pooled.

Isolation of Immunoglobulins. Antipneumococcal antibodies 2377, 2388, and K17 were isolated using a bromacetylcellulose immunoadsorbent (Pincus *et al.*, 1970b). K7 antibodies were isolated using a Sepharose immunoadsorbent (fraction 2A, Cheng and Haber, 1971). Normal immunoglobulins from serum pools of nonimmune rabbits, homozygous for the b_4 allotype, were prepared by two successive precipitations with 33% ammonium sulfate, followed by DEAE-cellulose chromatography (Levy and Sober, 1960).

Both antibody and nonimmune IgG were subjected to mild reduction with 0.1 M 2-mercaptoethanol, in 0.4 M Tris-HCl

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[1] Abbreviations used are: Pth-AA, phenylthiohydantoin derivative of a given amino acid; SCMC, sulfocarboxymethylcysteine; anti-SIII, anti-SVIII, antibodies directed against the type III or the type VIII pneumococcal polysaccharide. The nomenclature of and the abbreviations for the immunoglobulins and their subunits produced by reduction and alkylation are those recommended by the World Health Organization (1964).

buffer (pH 8.2) at 37° for 90 min. This was followed by alkylation with the addition of a solution of iodoacetamide in the same buffer in 10% molar excess over the 2-mercaptoethanol. The reaction was allowed to proceed for 15 min at 4°. The reaction mixture was then dialyzed against 1 M acetic acid for 18 hr with three changes of dialysate.

Light-Chain Preparations. Light and heavy chains were separated by gel filtration on Sephadex G-100 (Pharmacia) in 1 N acetic acid according to Fleischman *et al.* (1963).

Succinylation of nonimmune light chain was performed according to the procedure of Klotz (1967). Lyophilized light chain was dissolved in water and a 10-fold molar excess of crystalline succinic anhydride with respect to lysine was added gradually over 1 hr while the pH was maintained at 9.0 by the addition of 1 N NaOH.

Full reduction was carried out in 0.01 M dithiothreitol, 7 M guanidine·HCl, and 0.5 M Tris·HCl (pH 8.5) for 90 min at 37°. This was followed by the addition of iodoacetic acid in 10% molar excess over the dithiothreitol and the reaction was allowed to proceed for 15 min at 4°. [2-^{14}C]Iodoacetic acid (Amersham, Searle Co.) was added to yield a specific activity of 2–4 μCi/mg of light chain in reaction mixture.

Polyacrylamide disc gel electrophoresis was performed in 8 M urea at pH 9.5 as described previously (Pincus *et al.*, 1970a).

Hydrolytic Methods. Hydrolysis of fully reduced and alkylated light chains was performed with TPCK-treated trypsin (Worthington) in 1% ammonium bicarbonate (pH 8.2) at 37°. The initial substrate:enzyme ratio was 100:1. After 3 hr an equal amount of trypsin was added and the reaction was allowed to proceed an additional 3 hr.

Succinylated nonimmune light chain was subjected to acid hydrolysis in 10% acetic acid–pyridine (pH 2.5) in 7 M guanidine·HCl as described by Fraser *et al.* (1972).

The K7 light chain was hydrolyzed in 70% formic acid with a 5-fold excess by weight of CNBr for 24 hr at room temperature (Gross and Witkop, 1962). The solution was then diluted 10 times with water and lyophilized.

Amino acid compositions were determined on a Beckman analyzer, Model 120B, using the two-column system and an Infotronics digital integrator. Hydrolysates were prepared at 110° for 24 hr in vacuum-sealed tubes, containing 1 ml of constant-boiling HCl and 5 μl of butanedithiol to maximize the yield of tryptophan (80%) and sulfur containing amino acids (70%).

Separation of Peptides. Tryptic digests were chromatographed on Dowex 50-X2 (Bio-Rad). In a typical experiment, 100 mg of digest was dissolved in 0.2 M pyridine–acetate (pH 2.5) and applied to a 30 × 0.9 cm jacketed column, maintained at 37°. The column was developed by gradient elution from 0.2 to 2 M pyridine–acetate (pH 2.5 to 5). Stepwise elution with 2 M pyridine–acetate buffers (pH 6 and 8) and with 15% pyridine was used to recover peptides not obtained with the gradient.

Aliquots of all fractions were counted in Bray's solution (Bray, 1960) in a Packard liquid scintillation counter. Fractions containing radioactivity were lyophilized and then chromatographed on DEAE-Sephadex A-25 (Pharmacia). This chromatography was effected with a linear gradient from 0.005 to 1 M NH_4HCO_3 (pH 8.5) followed by a second gradient from 1 to 2 M NH_4HCO_3 and by stepwise elution successively with 15% pyridine, 0.5 N NaOH, and 2 M NaCl. Chromatography on SE-Sephadex C-25 (Pharmacia) was carried out with the same volatile pyridine–acetate buffer system as described for the Dowex 50 chromatography. The DEAE- and SE-Sephadex columns were of 5-ml bed volume and were contained in 10-ml syringes.

The CNBr digest of K7 light chain was dissolved in 1 N acetic acid and subjected to gel filtration on Sephadex G-100. The excluded fraction, (F1), thought to be an aggregate, was collected and lyophilized.

The purity of the peptides was monitored by high-voltage electrophoresis (Gilson) on Whatman No. 3MM paper using pyridine–acetate buffers at pH 3.6 and 6.5. Peptides were detected with the ninhydrin–cadmium stain (Dreyer and Bynum, 1967). Peptides were stained with Erlich's reagent (Smith, 1953) for the detection of tryptophan and radioactive peptides were revealed by radioautography, using Kodak No-Screen X-Ray film.

Desalting of peptides was accomplished on Bio-Gel P2, Sephadex G-10, or G-25. The developing solvents were 1 N formic acid, 1 N acetic acid, or 0.1 N NH_4OH, depending upon the solubility of the peptide. The conductivity of the eluent was measured to detect the salt fraction.

Sequence Analysis. Large fragments obtained using CNBr and acid hydrolysis and the intact light chain of K17 were sequenced using the protein sequenator (Beckman Sequencer 890B). The 1 M Quadrol program described by Edman and Begg (1967) was employed. The average repetitive yield in the sequenator was 95%. The smaller peptides were subjected to manual Edman degradation (Edman, 1970). Commonly between 0.25 and 1 μmol of peptide were used.

The Pth derivatives of the amino acid residues were identified by gas–liquid chromatography (Pisano and Bronzert, 1969) and by thin-layer chromatography (Edman, 1970). Silica gel coated glass plates were purchased from Analtech Co. (Delaware). An applicator manifold (Edman, 1970) was made available by Pierce Chemical Co. Solvents for systems D, E, and H (Edman, 1970) were obtained from commercial sources and used without furthur purification. The Pth derivative of ϵ-succinyllysine was detected by thin-layer chromatography. It migrates slower than Pth-asparagine in system H.

The detection of Pth-[^{14}C]SCMC was by gas chromatography (it chromatographs at the position of Pth-serine), by thin-layer chromatography, where Pth-SCMC runs close to aspartic and glutamic acids on solvent system H (Edman, 1970), and by radioautography of the thin-layer plate. At every step of the manual degradation a 2% aliquot was counted in Brays' solution in a Packard liquid scintillation counter.

In some instances the identity of Pth-amino acid derivatives was checked by amino acid analysis of hydrolysates (Van Orden and Carpenter, 1964).

Results

Heterogeneity of Light Chains. Peptides for sequence determination were obtained from light polypeptide chains of four antibody preparations: 2377, 2388 (both anti-SVIII), K7, and K17 (both anti-SIII) and from light chains from nonimmune γ-globulin.

Disc gel polyacrylamide electrophoresis (Figure 1) revealed a major degree of restriction of heterogeneity in the antibody light chains. The chains from K7 and K17 antibody contained one major component. Light chains from 2388 and 2377 contained two major and several minor electrophoretic species.

Additional evidence for homogeneity of the K17 light chain was obtained when only one amino acid was identified by automated Edman degradation at each of the N-terminal 11 positions (Figure 2).

Compositional analysis showed 6.2–6.6 mol of SCMC/mol

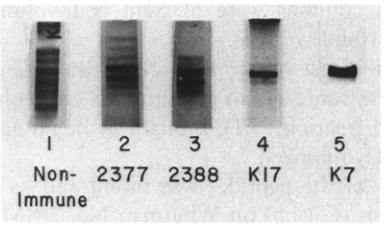

FIGURE 1: Polyacrylamide gel electrophoresis at pH 9.5 in 8 M urea of fully reduced and alkylated light chains: (1) nonimmune light chains, (2) light chains from 2377, (3) 2388, (4) K17, and (5) K7.

Chain	1								10
K17	Asp-	Val-	Val-	Met-	Thr-	Gln-	Thr-	Pro-	Ala-	Ser-	Val

FIGURE 2: Amino-terminal sequence of light chain K17.

of fully reduced and alkylated light chains for all four antibody preparations and nonimmune γ-globulin which indicated that they were of the κ_B type as defined by Rejnek *et al.* (1969). Where these authors separated κ_A and κ_B chains their analyses showed 4.8 half-cystine residues and 6.9 half-cystine residues for κ_B. The discrepancy from the theoretical values is probably related to oxidative losses.

Separation of the Major Radioactive Fractions of the Tryptic Digests. The tryptic digests of fully reduced and ^{14}C-alkylated light chains were chromatographed on Dowex 50. Very similar chromatograms were obtained with three antibody light-chain preparations and the preparation from nonimmune γ-globulin. A typical fractionation is represented in Figure 3.

Fraction 1 showed heterogeneity on rechromatography and subsequent sequence analysis. This mixture was not subjected to further study.

Fraction II of the Dowex 50 chromatogram of all preparations contained at least two peptides which separated poorly. Chromatography on DEAE-Sephadex did not improve their resolution but they could be further refractionated on Bio-Gel P2 (Figure 4). The compositions of the peptide IIb were identical for 3 preparations studied, including the one from nonimmune light chain (Table I).

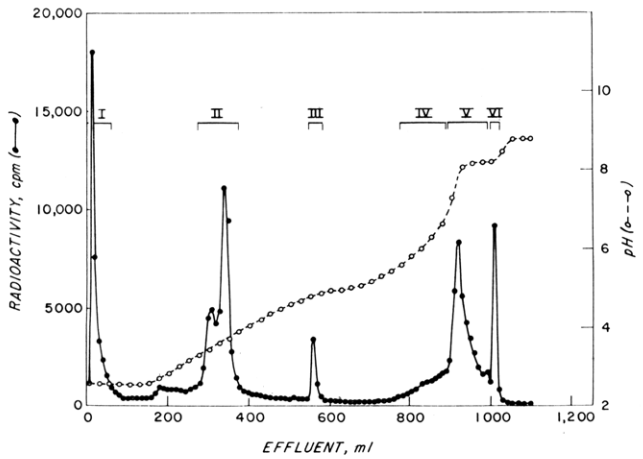

FIGURE 3: Dowex 50-X2 chromatography of tryptic digest from fully reduced and ^{14}C-alkylated light chain K17. Column dimensions: 30 × 0.9 cm jacketed column, maintained at 37°.

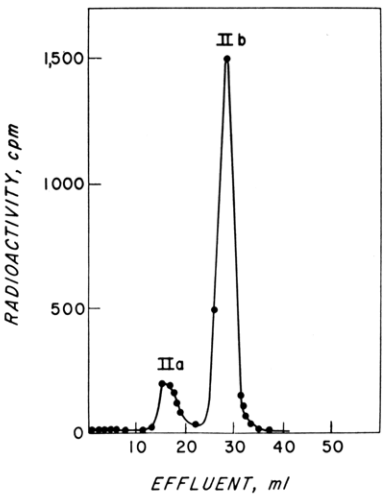

FIGURE 4: Gel filtration on Bio-Gel P2 of fraction II from the Dowex 50-X2 chromatography.

The sequences of the peptide IIb isolated from nonimmune light chains and from 2388 were obtained by sequential manual Edman degradation (Figure 5). After the fourth cycle ϵ-phenylthiocarbamyllysine was detected on the amino acid analyzer (Dowling and Stark, 1969).

The amino acid composition of peptide IIa from preparations 2377 and K17 is shown in Table II. These compositions were similar. The partial sequence of the peptide from preparation 2377 obtained by manual Edman degradation is shown in Figure 5. The corresponding fraction from nonimmune light chain and from 2388 showed two amino acids at each step of the manual Edman degradation (Figure 5), suggesting the presence of two peptides. Analysis of the mixtures by Edman degradation was necessary at this point (Niall and Potts, 1970) since insufficient material was available for further fractionation. Resolution of the mixtures was possible because the sequence of peptide IIa from 2377 was common to both (Figure 5). After subtraction of the sequence IIa, the fraction IIa from nonimmune pool showed a sequence similar to peptide IV of K17. The second component of peptide IIa from 2388 will be discussed later.

Fraction III of the Dowex 50 chromatogram was repurified on SE-Sephadex. Two radioactive fractions were obtained from all preparations. Fraction III_1, did not bind to the resin. Compositions of peptide III_1, for preparations 2377, K17, and nonimmune chains were given in Table III.

TABLE I: Peptide IIb.

Amino Acid	2388	K17	Nonimmune Light Chain
Lysine[a]	1.0 (1)	1.0 (1)	1.0 (1)
SCMC	0.9 (1)	0.7 (1)	0.9 (1)
Threonine	0.9 (1)	0.8 (1)	0.9 (1)
Glutamic acid	1.1 (1)	1.0 (1)	1.0 (1)
Tyrosine	0.8 (1)	0.9 (1)	0.9 (1)
Sum	(5)	(5)	(5)
Yield of peptide (%)	20	36	50

[a] Lysine was taken to be 1.

L Chain	Peptide	Peptide Sequences (1 5 10 15)
2377	IIa	Thr-Pro-Gln-Asn-Ser- Ala-Asp-Cys-Thr-Tyr- Val-Leu-Ser- Ser- Thr-Leu-Thr-Leu
2388	IIa	Thr Pro Gln Asn Ser Ala Asp Cys Thr Tyr Val Leu Ser Ser Thr Leu Thr Leu Tyr Phe Pro Asp Val Thr Val Thr Ser Glu Asp Gly Thr Gln
Nonimmune	IIa	Thr Pro Gln Asn Ser ( ) Asp Cys Thr Tyr Leu Leu Cys Gln Ala Ser Glu ( ) Ile ( ) Ser Ala
K17	IV_1	Cys-Gln-Ala-Ser- Glu-Ser- Ile- Tyr-Ser
2377	IV_1	Cys-Gln
2288	IIb	Glu-Tyr-Thr-Cys-Lys
Nonimmune	IIb	Glu-Tyr-Thr-Cys-Lys

FIGURE 5: Peptide sequences from peptides IIa, IV_1, and IIb. () Not identified.

The sequences of the peptides III_1 from 2388 and 2377 are shown in Figure 6 as obtained by direct manual Edman degradation. Twenty-eight steps were performed on the thirty-one residue peptide III_1 (2377). An analysis of yield at each step is shown in Figure 7. Peptide III_1 (2388) was only partially sequenced, but appears identical.

The partial sequences of the cyanogen bromide fragment F1 from K7 and the product of acid hydrolysis of succinylated nonimmune light chain (Suc-2) are also shown in Figure 6. These were obtained using the protein sequenator. The fractionation of Suc-2 from cleaved light chain prior to Edman degradation was unnecessary because of acylation of the amino terminus of the light chain during the succinylation procedure.

Fractions IV and V of the Dowex 50 chromatogram were also subjected to SE-Sephadex chromatography and a number of fractions were obtained. Peptides IV_1 from K17 and 2377 were partially sequenced and are shown in Figure 5.

The yield of fraction V from nonimmune globulin was 4% and the Edman degradation showed 2 amino acids at each step. It was possible, however, to resolve the mixed sequence since at each step the yield of each of the two Pth-amino acids was different. Figure 8 shows quantitative yields of Pth-amino acids at each step plotted on a semilogarithmic scale. Two discrete curves could be plotted. The tentative sequences V_1 and V_2 represented by the upper and lower curves, respectively, are shown in Figure 9.

Discussion

Although the sequence analysis of rabbit antibody light chain is as yet incomplete, a number of interesting conclusions may be drawn from an examination of the sequences of several of the cysteine containing peptides. It is apparent that extensive sequence homology exists between rabbit and human light chains. This allows for tentative placement and ordering of the peptides sequenced in this study and identification thereby of the position of the third intrachain disulfide bridge, which has not been found in other species thus far studied. Variability in constant region sequences among different chains could also be inferred, suggesting multigenic origin of a light chain of single allotype.

Figure 10 aligns the peptide sequences IIa and IIb with the constant region of human light-chain sequence Ag (Putnam *et al.*, 1966). Marked homology is apparent, with 12 of 14 residues identical between peptides IIa and IIb and Ag between positions 164 and 181. Rabbit light chains contain but one histidine, which may be homologous with position 189 in Ag as this residue has been found in all mammalian light chains sequenced thus far. This may confirm our placement of

TABLE II: Peptide IIa.

Amino Acid	2377	K17
Lysine[a]	1.0 (1)	1.0 (1)
Histidine	0.6 (1)	0.5 (1)
SCMC	0.6 (1)	0.7 (1)
Aspartic acid	3.0 (3)	3.2 (3)
Threonine	3.6 (4)	4.3 (4)
Serine	2.9 (3)	2.7 (3)
Glutamic acid	2.3 (2)	2.8 (3)
Proline	0.9 (1)	1.0 (1)
Glycine	0.8 (1)	1.1 (1)
Alanine	0.9 (1)	0.8 (1)
Valine	1.0 (1)	0.7 (1)
Isoleucine	0.5 (1)	0.5 (1)
Leucine	1.6 (2)	2.1 (2)
Tyrosine	1.3 (1)	1.1 (1)
Sum	(23)	(24)
Yield of peptide (%)	16	23

[a] Lysine was taken to be 1.

TABLE III: Peptide III_1.

Amino Acid	2377	K17	Nonimmune Light Chain
Lysine[a]	1.0 (1)	1.0 (1)	1.0 (1)
SCMC	1.0 (1)	0.7 (1)	0.6 (1)
Aspartic acid	2.9 (3)	2.7 (3)	2.9 (3)
Threonine	3.8 (4)	3.6 (4)	3.8 (4)
Serine	0.5 (1)	0.5 (1)	0.8 (1)
Glutamic acid	1.3 (1)	1.6 (2)	1.7 (2)
Proline	4.0 (4)	3.8 (4)	4.0 (4)
Glycine	1.8 (2)	2.3 (2)	2.0 (2)
Alanine	4.5 (5)	4.1 (4)	4.5 (5)
Valine	5.8 (6)	4.9 (5)	5.1 (5)
Isoleucine	1.5 (2)	1.5 (2)	1.8 (2)
Leucine	1.1 (1)	1.0 (1)	1.1 (1)
Phenylalanine	0.7 (1)	0.8 (1)	0.8 (1)
Sum	(32)	(31)	(32)
Yield of peptide (%)	22	15	4

[a] Lysine was taken to be 1.

b_4 Rabbit Light Chain (93–152)

L Chain	Peptide	Position 93	100	110
Ag		Thr-Leu-Pro-Arg-Thr-Phe-Gly-Gln-Gly-Thr-Lys-Leu-Glu-Ile-Lys-Arg-Thr-Val-Ala-Ala-Pro-Ser-Val-Phe-Ile-Phe-Pro-		
K7	F1	Ile-Thr-Ala-Thr-Thr-Phe-Gly-Gly-Gly-Thr-Glu-Val-Val-Val-( )-Gly-Asp-Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-( )-		
2388	III_1			Gly-Asp-Pro-Val-Ala-( )-Thr-Val-Leu-Ile-Phe-( )-
2377	III_1			Gly-Asp-Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-
2388	IIa			
Nonimmune	Suc 2			Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-

FIGURE 6: Peptide sequences from peptide III_1, F1 (K7), and Suc-2 (nonimmune). These sequences are compared with that of protein Ag

peptide IIa which contains a single histidine by compositional analysis. Also presented in this figure are peptides sequenced by Appella and coworkers (1971), which can now be located. This places Cys-194 in the rabbit light chain in a position analogous with that in human and murine light chains. The additional half-cystine not seen in human or murine chains is present at position 171.

Peptide III_1, aligns with Ag between positions 108 and 138 (Figure 6), where 14 of 31 residues are identical. This places Cys-134 in a position homologous with that of other mammalian light chains sequenced. Two other lines of evidence support this assignment. The first is the sequence of an overlapping peptide from chain K7, obtained during cyanogen bromide cleavage which shows 13 of 34 identities with Ag from positions 93 to 126. The second line of evidence is derived from studies on dilute acid hydrolysis of antibody light chains which has been shown to result in a specific cleavage in the switch region (Fraser *et al.*, 1972). The sequence of fraction Suc-2 produced in this manner and presented in Figure 6 shows identity with positions 110–135 of peptide III_1, indicating that this peptide is indeed located at the beginning of the constant region.

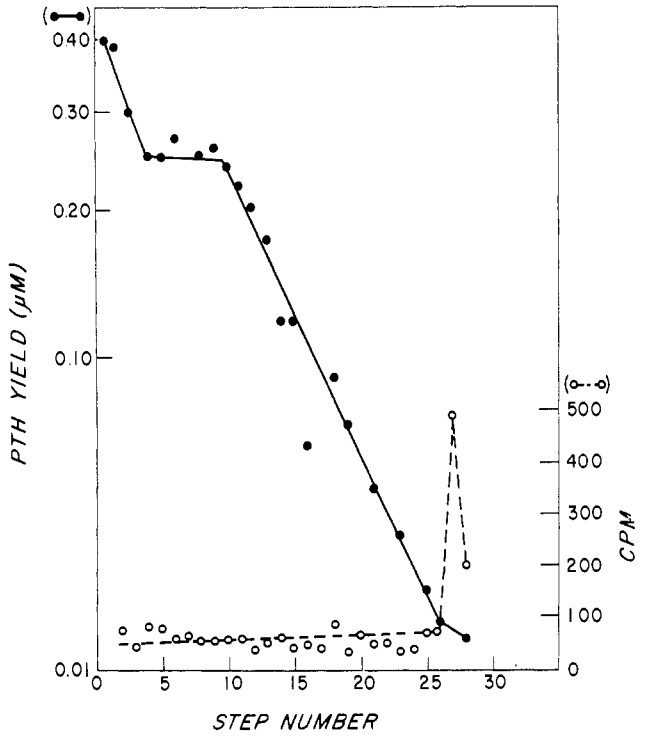

FIGURE 7: (●) Yields at each step in the manual Edman degradation of peptide III_1 (2377). Quantitative yields of Pth-amino acids were computed by comparing the peak heights of the sample with the relevant standard Pth derivative. (O) cpm of a 2% aliquot of the Pth-amino acid at each step. Step 27 indicates SCMC.

The residues between positions 139 and 146 in Suc-2 are identical with the second component of mixture IIa from 2388 light chain. The same sequence was also found in positions 2–8 in peptide 2C previously reported by Freedlender and Haber (1972). This peptide of 67 residues occupies positions 140–207 of the light chain according to our results. The amino acid composition of peptide 2C includes 2 residues of sulfocarboxymethylcysteine which we have now located at positions 171 and 194.

Peptide IV_1 which has an amino-terminal cysteine may be placed at position 23, as lysine has been found in all antipneumococcal light chains sequenced to that position. There is also homology with Ag (5 of 9 positions) (Figure 11).

Peptide V_1 is highly homologous with positions 62–77 of Ag (11 of 16 positions) (Figure 12). V_2 cannot be readily placed by homology, though on the basis of its size it must lie in the variable region. From present and previous data (Freedlender and Haber, 1972) the composition of the constant region is entirely accounted for. The available gaps in the variable region, therefore, place peptide V_2 somewhere between positions 40 and 85.

A peptide corresponding to the region containing Cys-88 of other species has not been identified in this study.

All mammalian light chains sequenced thus far have two intrachain disulfide bridges, one within the variable and one

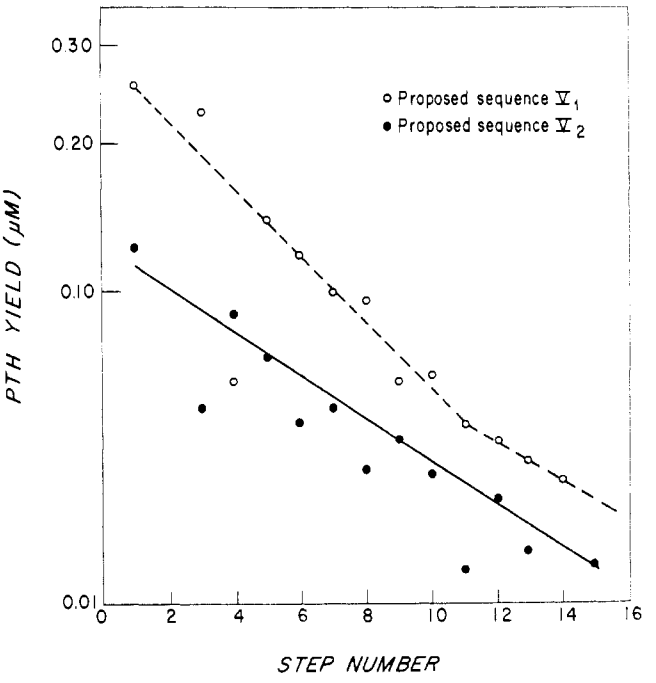

FIGURE 8: Quantitative yields of Pth-amino acids obtained at each step of the manual degradation of fraction V. See Figure 7 for method of calculating yields. PTH-Ser at step 4 was assigned to sequence V_1, as it is usually recovered in low yield.

120 130 134 140 150

Pro-Ser-Asn-Glu-Gln-Leu-Lys-Ser-Gly-Thr-Ala-Ser-Val-Val-Cys-Leu-Leu-Asn-Asn-Phe-Tyr-Pro-Arg-Glu-Ala-Lys-Val-Gln-Trp-Lys-Val-Asp-Asn

Pro-Ala-Ala-Asp-( )-Val-Ala

Pro-Ala-Ala

Pro-Ala-Ala-Asp-Gln-Val-Ala-Thr-Gly-Thr-Val-Thr-Ile-Val-Cys-Val(Ala Asx)Lys

Tyr-Phe-Pro-Asp-Val-Thr-Val-Thr-Ser-Glu-Val-Asp-Gly-Thr

Pro-Ala-Ala-Asp-Gln-Val-Ala-Thr-Gly-Thr-Val-Thr-Ile-Val-( )-Val-Ala-Asn-Lys-Tyr-Phe-Pro-Asp-Val-Thr-Val-Thr

between residues 93 and 152. () Not identified; (Ala Asx) not positioned.

within the constant regions, respectively. These bridges are located from 23 to 88 and 134 to 194 in Ag and in homologous positions in all other chains. Lamm and Frangione (1972) have isolated the corresponding cystine peptides confirming the existence of these disulfide bridges in the rabbit light chain. It is likely that the additional Cys peptides (IIa and V_2) described in this study represent the third intrachain disulfide bridge from the κ_B chains, which extends between position 171 in the constant region and the variable region. Corresponding cystine-containing peptides have also been isolated by Lamm and Frangione (1972) and support our assignment.

Further compelling evidence of a disulfide bridge between variable and constant domains is adduced from the demonstration that peptide bond cleavage at the switch region does not result in reduction of the Stokes radius of the light chain until it has been subjected to complete reduction and alkylation (Poulsen *et al.*, 1972).

The association of at least 3 sets of variable regions with the same constant region in human κ chains has been interpreted as indicating that an individual light polypeptide chain is the product of two genes, one coding for the constant region, the other for the variable region. The genome is thought to contain at least 3 but probably many more genes coding for variable regions (Milstein, 1967; Hood *et al.*, 1967). The demonstration in rabbits of a disulfide bridge connecting the variable and constant regions now relates a structural feature in one part of the polypeptide chain to the other and places a constraint on possible mutation in the variable region. It is apparent that each member of the library of genes coding for κ_B variable regions in the rabbit must have a codon for a third cysteine and if mutation occurs at this position, then the gene product can no longer be used in association with the κ_B constant region. A class of light chains possessing a free sulfhydryl group in the constant region has not been recognized in rabbits but three myeloma protein λ light chains possessing a free SH group in the variable region have been found (Milstein *et al.*, 1968; Baczko *et al.*, 1970; Buchwald *et al.*, 1971). It would be interesting to determine where in the scale of evolution the third disulfide bridge first appeared. If the extra bridge is shown to lie symmetrically this would be in accordance with the evolution of the variable and constant region genes from a primordial gene by gene duplication. In this case, one may expect to find such an extra disulfide bond in more primitive species.

Edelman *et al.* (1969) have proposed a domain hypothesis for the organization of the immunoglobulin molecule in which the variable and constant halves of the light chain are envisioned as part of two dense and independently folded units. This is supported by the recent X-ray crystallographic model of Poljak *et al.* (1972). The presence of an interdomain disulfide bridge might be viewed as creating structural constraints between these domains. Indeed, Edelman (1970) has suggested that such a bridge may be inconsistent with his hypothesis. However, there is little evidence for independent rotation of these domains in polarization of fluorescence studies (Yguerabide *et al.*, 1970), which indicate that the entire Fab fragment behaves as a rigid ellipsoid in solution. A comparison of the crystallographic structures of trypsin and chymotrypsin indicates that the presence of two additional disulfide bonds in trypsin does not tend to distort markedly the three-dimensional structure of this molecule in comparison to chymotrypsin which does not possess these bonds. This suggests that while the light-chain inter-domain disulfide bond may stabilize conformation, it may not alter it in comparison with light chains which do not possess this bond. Optical rotatory dispersion studies (Björk and Tanford, 1971) have shown that rabbit light chains are more resistant to denaturation at low pH than are human light chains. This may be consistent with structural stabilization provided by an interdomain disulfide bridge.

A comparison of our data with that of Frangione and Lamm (1971) shows that position 169 is alanine in b_4 nonimmune light chains as well as in antibody chains whereas it is aspartic acid in b_5 nonimmune chains. As Appella *et al.* (1969) and Frangione (1969) have shown allotype related sequence differences between positions 210 and 214, this would indicate that allotype related residues are likely to be located in more than one part of the constant region. In contrast, the Inv allotype in human κ chains is represented by a single amino acid interchange (Baglioni *et al.*, 1966).

It is also interesting to note that whereas two antibody light chains had a valine residue at position 174, this position is occupied by leucine in the nonimmune light chains reported here and in reports of Appella *et al.* (1971) and Lamm and Frangione (1972). This valine–leucine interchange indicates the existence of at least two sequences among the b_4 light-chain populations. This suggests at least two genes within a single allele of a single allotype in κ_B light chains. It is difficult to reconcile this finding with the simple Mendelian inheritance of the b allotypes unless recombination is excluded by some genetic mechanism. The expression of more than 2 allotypes in a single rabbit has not thus far been observed. An

Manual Edman Degradation Fraction V

Residues Found		Phe ( ) Gly Ser Gly Ser Gly Thr Gln Phe Thr Leu Thr Ile Ser Asp Gly Pro Glu Thr Leu Phe Leu Leu Ser Glu Cys Ala
Proposed Sequences	V_1	Phe-( )-Gly-Ser- Gly-Ser- Gly-Thr-Gln-Phe- Thr-Leu-Thr-Ile- Ser- Asp
	V_2	Gly-( )-Pro-Glu-Gly-Thr-Leu-Phe-Leu-Leu-Ser- Leu-Glu-Cys-Ala

FIGURE 9: Sequences from peptides contained in fraction V. () Not identified.

b_4 Rabbit Light-Chain Constant Region (162–198)

L Chain	Peptide	Position 162	170	180	190	198
Ag		Ser-Val-Thr-Glu-Gln-Asp-Ser-Lys-Asp-Ser-Thr-Tyr-Ser-Leu-Ser-Ser-Thr-Leu-Thr-Leu-Ser-Lys-Ala-Asp-Tyr-Glu-Lys-His-Lys-Val-Tyr-Ala-Cys-Glu-Val-Thr-His				
2377, 2388	IIa	Thr-Pro-Gln-Asn-Ser-Ala-Asp-Cys-Thr-Tyr-Val-Leu-Ser-Ser-Thr-Leu-Thr-Leu				
Nonimmune	IIa	Thr-Pro-Gln-Asn-Ser-Ala-Asp-Cys-Thr-Tyr-Leu-Leu-Ser-Ser-Thr-Leu-Thr-Leu			Glu-Tyr-Thr-Cys-Lys	
2388	IIb					
Nonimmune[a]	1-2	Ser-Lys-Thr-Pro-Glu-Asp-Ser-Ala-Asp-Cys-Thr-Tyr				
2717[a]	2-2	Asp-Cys-Thr-Tyr-Leu				
Nonimmune[a]	1-1				Thr-Ser-Thr-Glu-Tyr-Asx-Ser-His-Lys-Gln-Tyr-Thr-Cys-Lys-Gly-Thr-Val	

[a] Appella *et al.* (1971)

FIGURE 10: The b_4 rabbit light-chain sequence constant region between positions 162 and 198 as compared with the sequence of protein Ag.

b_4 Rabbit Light-Chain Sequence from Position 62 to 77

L Chain	Peptide	Position 62	70	77
Ag		Phe-Ser-Gly-Ser-Gly-Phe-Gly-Thr-Asp-Phe-Thr-Phe-Thr-Ile-Ser-Gly		
Nonimmune	V_1	Phe-( )- Gly-Ser-Gly-Ser- Gly-Thr-Gln-Phe-Thr-Leu-Thr-Ile-Ser-Asp		

FIGURE 12: The b_4 rabbit light-chain sequence between positions 62 and 77 as compared with the corresponding region of protein Ag.
( ) Not identified.

b_4 Rabbit Light-Chain Sequence from Position 23 to 31

L Chain	Peptide	Position 23	30
Ag		Cys-Gln-Ala-Ser-Gln-Asx-Ile-Asx-Ser	
2377	IV_1	Cys-Gln	
K17	IV_1	Cys-Gln-Ala-Ser-Glu-Ser-Ile-Tyr-Ser	
Nonimmune	IIa	Cys-Gln-Ala-Ser-Glu-Ser-Ile	

FIGURE 11: The b_4 rabbit light-chain sequence between positions 23 and 31 as compared with the corresponding region of protein Ag.

alternative explanation is the possible lack of specificity of b_4 antisera. These antisera may fail to differentiate between the phenotypic products of two allelic genes at the b locus.

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Conformational Variation in a Human Plasma Lipoprotein†

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ABSTRACT: A specific and homogeneous α-2 globulin (a lipoprotein) has been repeatedly isolated from plasma of individual human subjects. The α-2 globulin samples are the same in amino acid and lipid composition, in chromatographic elution properties, and in electrophoretic mobility; but they do differ in their effects on a number of intermediary metabolic processes, in their effect on intracellular tryptophan transport, and in their *in vivo* effects on trained rats. In measurements of the optical rotatory dispersion (ORD) and circular dichroism (CD) of these lipoproteins, differences in conformation were found in a given subject from time to time, and between samples from paired subjects studied at the same time. Among the protein samples from patients with schizophrenia, various amounts of α-helical conformation with f_H up to 0.74 were found, with all patients having their protein in this conformation on one or more occasions. Among α-2 globulin samples from healthy control subjects, either the β and/or random-chain conformation was found. The differences in biochemical and biological activities among these α-2 globulin samples seem to be related to the differences in conformation.

In physicochemical studies on samples of an α-2 globulin isolated from individual subjects, measurements of optical rotatory dispersion (ORD) and circular dichroism (CD) were made. Significant differences in conformation were found, and there was evidence for conformational flexibility. The results of these conformation studies for 79 individual samples of the α-2 globulin, prepared from the plasmas of 30 human subjects, are presented in this report.

Several classes of human lipoproteins have been reported to undergo reversible conformation changes *in vitro* as the temperature was altered, with more α-helical conformation at 0–5°, and with increased β conformation at 37° and above (Scanu *et al.*, 1969; Dearborn and Wetlaufer, 1969). There have been reports that other protein molecules with various amounts of α-helical content can be changed reversibly *in vitro* to the random-chain or the β conformation. Among these proteins are serum albumin (Kolthoff *et al.*, 1960), ribonuclease (Anfinsen, 1962; Epstein *et al.*, 1963), and myoglobin and apomyoglobin (Harrison and Blout, 1965). From studies of the transition temperature (T_m) for proteins as the molecular (ionic) environment was varied, von Hippel and Schleich (1969) concluded that most proteins have "marginal conformational stability," and that folded macromolecules were in a dynamic equilibrium with various unfolded forms. From hydrogen-exchange studies under physiological conditions, others have also concluded that folded native molecules were in equilibrium with unfolded molecules (Hvidt and Nielsen, 1966; Rosenberg and Chakravarti, 1968). These conformational changes are reported to be thermodynamically feasible because the net ΔF/mol is small (Nemethy *et al.*, 1963; Lumry and Biltonen, 1969).

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