A Method for Obtaining Large Variable-Region Peptides from Rabbit Light Chains†

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ABSTRACT: Definition of the relationship between antibody specificity and primary structure requires that the variable regions of many antibodies be sequenced and compared. Obtaining the necessary sequence data would be facilitated by a method for reproducibly isolating large fragments of variable regions suitable for automatic Edman degradation. The availability of rabbit antibodies of restricted heterogeneity increases the feasibility of achieving this goal. This communication reports the development of such a method for the rabbit light chain. Light chains were isolated from an antipneumococcal polysaccharide antibody of restricted heterogeneity from a single rabbit homozygous for the b_4 light-chain allotype. Disc gel electrophoresis indicated that the antibody preparation contained four distinguishable electrophoretic species of light chain. After complete reduction and alkylation with [^{14}C]iodoacetic acid to label the cysteines involved in the intrachain disulfide bonds, the light chains were maleylated and digested with trypsin. Gel filtration separated the digest into a fraction containing the constant region, two containing variable-region peptides and one containing the carboxyl-terminal tripeptide. Ion-exchange chromatography resolved the major fraction containing variable-region peptides into the amino-terminal peptides and two other variable-region peptides. The amino-terminal peptides of about fifty residues were isolated as a single fraction. The antibody derived peptides had amino-terminal sequences identical with those of the original light-chain preparation. Peptide maps of the chymotryptic digest of this fraction indicated that several constant region peptides were missing and the amino acid composition was different from that of whole light chain. Two other variable-region peptides of about 65 residues were isolated using the same procedure. One of these had Phe-Ser-Gly-Ser-Gly as its amino-terminal sequence. This sequence is characteristic of all κ light chains and occurs following Arg-62. The other peptide had an amino-terminal sequence which could not be placed by homology with any of the reported mouse or human sequences. Peptide maps suggested that these two peptides included homologous stretches and continued into the constant region. The antibody light chain used as starting material contained several sequences as evidenced by more than one amino acid released at each step of the Edman degradation. In the amino-terminal peptide fraction isolated after limited cleavage, the same residues were again present. This indicates that the amino-terminal peptides of all the chains initially present were not separated from each other, although they were separated from non-amino-terminal peptides. This suggests that the method should be generally applicable to isolating the homologous peptides from any rabbit antibody.

The availability of rabbit antibodies of restricted heterogeneity from which a homogeneous species may be isolated (Krause, 1970; Cheng and Haber, 1971) makes it feasible to study the relationship between antibody specificity and primary structure. For this type of investigation, the variable regions of many antibodies need to be sequenced and compared. Obtaining the necessary sequence data would be facilitated by a method for reproducibly isolating large fragments of variable regions suitable for automatic Edman degradation. This communication reports the development of such a method for the rabbit light chain.

Rabbit light chains of the b_4 allotype contain an average of three arginines (Reisfeld and Inman, 1968; Appella *et al.*, 1970). One of these has been shown to be the fourth residue from the carboxyl terminus (Appella *et al.*, 1970; Frangione and Lamm, 1970). Appella *et al.* (1970) have proposed that the other two arginines are located in the variable region. The few number of arginines and their tentative locations suggested that cleavage at arginines would be a convenient method for producing variable-region peptides suitable for automatic Edman degradation. The high degree of specificity of trypsin for arginine and lysine (Smyth, 1967) and the availability of specific blocking agents for lysine increased the attractiveness of this approach.

From a survey of reversible blocking agents for lysine, maleic anhydride was selected as the most promising. Conditions for complete masking and unmasking of light-chain lysines were established. Tryptic cleavage of maleylated light chains produced a small number of peptides which were easily separated by gel filtration and ion-exchange chromatography. Characterization of these peptides indicated that one fraction contained the amino-terminal peptides which continued through the first hypervariable region. A second fraction consisted of a peptide which started at Phe-62 and continued through the third hypervariable region.

Materials and Methods

Materials. Specific antibodies against type III pneumococcal polysaccharide were raised in a homozygous b_4 rabbit (90) and isolated as described by Jaton *et al.* (1970). Nonantibody γG-globulin was isolated by DEAE-cellulose chromatography from a pool of sera obtained from b_4 homozygous rabbits.

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Iodoacetic acid (Eastman Kodak) was recrystallized before use. [^{14}C]Iodoacetic acid was obtained from Amersham–Searle, ^{125}I-labeled hormones and [^{131}I]Na from Cambridge Nuclear Radiopharmaceutical Corp. Maleic anhydride was obtained from Aldrich Chemical Co., *S*-Ethyl trifluorothioacetate from Eastman Kodak and tetrafluorosuccinic anhydride from Pierce Chemical Co. Chymotrypsin (twice recrystallized) and trypsin (α-(1-tosylamido-2-phenyl)ethylchloromethyl ketone treated) were Worthington Biochemical Corp. products. G-10, G-50, and G-75 Sephadex and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals, Inc., and prepared according to the manufacturer's recommendations. Reagents for Edman degradations were obtained from Beckman Instruments. NH_4OH was redistilled before use by the method of Hirs (1967). Urea solutions were deionized with Amerlite MB-1 immediately before use. All other chemicals were reagent grade; distilled, deionized water was used throughout.

Modification of Lysine. Modifications were carried out in a pH-Stat (Radiometer) which delivered standard base from a calibrated syringe. Fully reduced and alkylated pool light chains were reacted with tetrafluorosuccinic anhydride as described by Braunitzer *et al.* (1968) and *S*-ethyl trifluorothioacetate as described by Goldberger and Anfinsen (1962). For maleylation fully reduced and alkylated light chains were either dissolved directly in sodium borate buffer (0.166 M H_3BO_3–0.133 M NaCl, adjusted to pH 9.0 with 10 M NaOH (Freedman *et al.*, 1968)) or dissolved in freshly deionized 10 M urea and then diluted fivefold with borate buffer. The solution was cooled to 0°. Maleic anhydride was added as a 1 M solution in dioxane while the pH was automatically maintained at 9.0 ± 0.1 by addition of 5 M NaOH. The reaction mixture was stirred 30 min at 0° and dialyzed exhaustively *vs.* 0.1% NH_4HCO_3 (pH 8.0).

Demaleylation was accomplished by incubating at 40° maleylated light chains in either 10% acetic acid–5 M guanidine·HCl (pH 2.5) or 10% acetic acid–1% pyridine (pH 3.5).

In early experiments the number of blocked lysines was assessed by the trinitrobenzenesulfonic acid method of Habeeb (1966). As this proved too insensitive to detect low levels of unblocked lysines, the dinitrophenylation (DNP) method of Schroeder and LeGette (1953) was used in later experiments. Esterification of hydroxyamino acids was assessed by the hydroxylamine–$FeCl_3$ method of Hestrin (1949) and esterification of phenolic hydroxides by the method of Riordan and Vallee (1964).

Tryptic Digestion. Completely maleylated antibody light chains (130 mg) which had previously been fully reduced and alkylated with [^{14}C]iodoacetic acid were dissolved in 0.1% NH_4HCO_3 and digested with trypsin at 37°, the pH being maintained at 8.0 ± 0.1 by the addition of 1 M NaOH with the pH-Stat. Three aliquots of trypsin in a 1:100 enzyme to substrate ratio were added at 30-min intervals. After a total of 3-hr digestion, the reaction was terminated by addition of 1 μg of DFP in 100 μl of isopropyl alcohol and the mixture was then lyophilized.

Fractionation of Peptides. The tryptic digest was separated by gel filtration on a 2.5 × 184 cm column of G-50 Sephadex in 6 M urea–0.1 M acetic acid. After demaleylation the major methionine-containing fraction was further fractionated on a 4-cm^3 column of DEAE-Sephadex A-25. Protein (50 mg) was dissolved in 5 ml of 0.005 M NH_4HCO_3; the pH was adjusted to 8.5 and the solution was diluted to the starting conductivity of 0.46 mho. The solution was loaded on the column equilibrated in 0.005 M NH_4HCO_3 (pH 8.5) and the column was washed with 100 ml of the same buffer. The column was eluted in a convex gradient with 1000 ml of NH_4HCO_3 (pH 8.5) from 0.005 to 0.4 M. A second convex gradient ranged between 0.3 and 1.0 M. At the end of the gradients, the column was stripped with 100 ml of 1 M NH_4OH followed by 100 ml of 0.5 M NaOH–2 M NaCl.

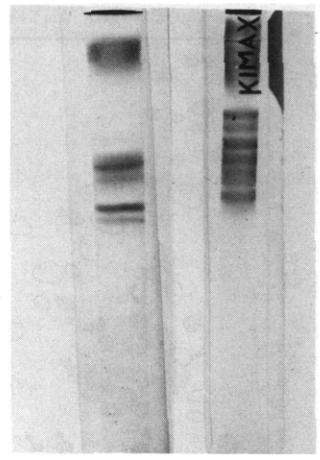

FIGURE 1: Alkaline urea gel electrophoresis of fully reduced and alkylated γG-globulin preparations. Antibody 90 is on the left, the pool on the right. The light chains migrated through about one-third of the gel; the heavy chains remained at the top. Anode is at the bottom.

The fraction containing small peptides was further purified by high-voltage paper electrophoresis for 40 min at 60 V/cm in pyridine–acetate buffer (pH 6.5). Standards were run simultaneously on a separate paper. The area migrating with dinitrophenyl-*S*-carboxymethylcysteine (DNP-SCMCys) was cut out and eluted with 1 M NH_4OH.

Peptides were desalted on G-10 Sephadex equilibrated in 1 M NH_4OH and lyophilized.

Other Methods. Isolation of light chains, full reduction and alkylation, alkaline disc gel electrophoresis, peptide mapping, and radioautography were carried out as described (Jaton *et al.*, 1970). In light-chain preparations labeled with [^{14}C]iodoacetic acid, the specific activity was approximately 2 μCi/mole of cysteine.

Amino acid analysis was performed as described by Spackman *et al.* (1958) on a Beckman 121 analyzer equipped with a high-sensitivity cuvet and digital integrator. Samples were hydrolyzed under reduced pressure in constant-boiling HCl at 100° for 20 hr. All hydrolyses were done in the presence of 0.5% (v/v) 1,4-butanedithiol to protect methionine, tryptophan, and SCMCys. Tryptophan was recovered in 80% yield.

^{14}C radioactivity was measured by liquid scintillation counting in Bray's (1960) solution in a Packard TriCarb spectrometer. Specific activities were determined using a [^{14}C]toluene internal standard. ^{131}I and ^{125}I were counted in a double-channel Nuclear-Chicago γ counting system.

Amino-terminal sequences were determined by the manual Edman technique (Edman, 1950) as modified by Niall and Potts (1970). 3-Phenyl-2-thiohydantoin (PTH) amino acids were identified by gas–liquid chromatography (Pisano and Bronzert, 1969) and amino acid analysis after conversion of PTH derivatives to free amino acids by hydrolysis in 57% HI for 20 hr at 125°. Serine and threonine are identified as alanine and aminobutyric acid in this procedure.

Molecular weights were determined by gel filtration (Whitaker, 1963) on 1 × 190 cm columns of G-75 Sephadex equili-

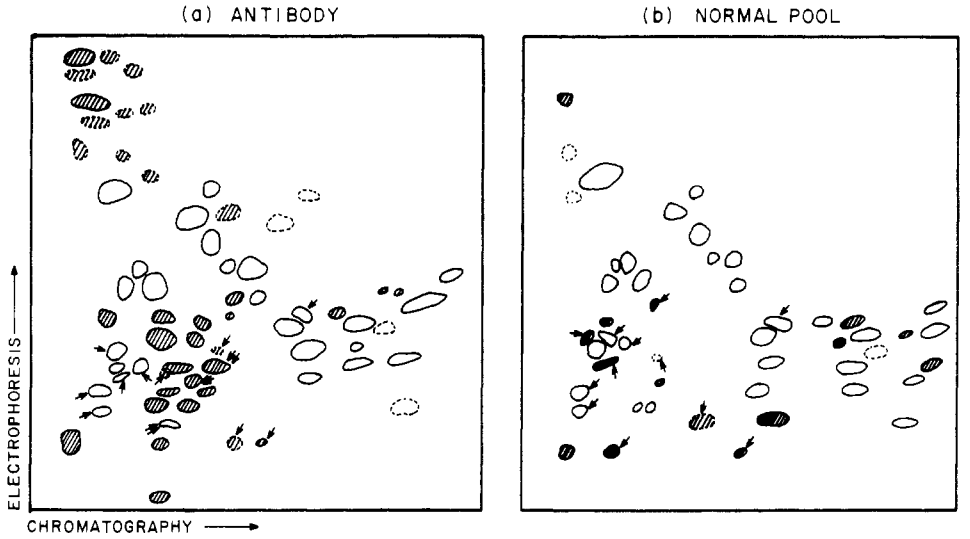

FIGURE 2: Schematic drawings of peptide maps of chymotryptic digests of light chains which were fully reduced and alkylated with [^{14}C]iodoacetic acid. Variable peptides are indicated by shading. Radioactive peptides are denoted by arrows. The three peptides with double arrows in the antibody map are the unique cysteine-containing peptides.

TABLE I: Modification of Lysines.

Reagent	Conditions	Molar Ratio Reagent:NH_2 Groups	% Blocked Lys	Assay
Tetrafluorosuccinic anhydride	7 M Urea, 12 mg/ml, 0°	3:1	25	TNBS[a]
	7 M Urea, 5 mg/ml, 0°	10:1	50	TNBS
	7 M Urea, 5 mg/ml, 0°	20:1	50	TNBS
S-Ethyl trifluorothioacetate	H_2O, 4.6 mg/ml, 25°	500:1	50	TNBS
	8 M Urea, 3 mg/ml, 25°	500:1	50	DNP
Maleic anhydride	Borate buffer, 15 mg/ml, 0°	100:1	35	DNP
	Borate buffer, 15 mg/ml, 0°	150:1	80	DNP
	Borate buffer, 15 mg/ml, 0°	200:1	82	DNP
	2 M Urea + borate buffer, 4–10 mg/ml, 0°	200:1	>95	DNP

[a] TNBS = trinitrobenzenesulfonate.

brated in 6 M urea, 0.1 M acetic acid. Peptides were iodinated with ^{131}I by the method of Greenwood *et al.* (1963) and chromatographed simultaneously with standards: cytochrome *c*, blue dextran, ^{125}I growth hormone, [^{125}I]parathyroid hormone and [^{125}I]glucagon.

Results

Rabbit 90 Light Chain. Light-chain-banding patterns obtained by disc gel electrophoresis of fully reduced and alkylated antibody 90 and normal γG-globulins from a b_4 pool are shown in Figure 1. The antibody light chains were distributed in two major bands with two minor bands and some diffuse background material. The antibody preparation clearly contained fewer electrophoretic species than did the normal γG-globulins.

Another indication of decreased heterogeneity was provided by peptide mapping. The map of the antibody light chains contained several spots not present in maps of the b_4 pool light chains (Figure 2). These probably represented variable-region peptides which were present in too low concentrations to be seen in the pool. A similar comparison has been made by Jaton *et al.* (1970). Radioautographs of these maps revealed that the antibody preparation contained three radioactive peptides not found in the pool. These were inferred to be variable-region cysteine peptides. The presence of variable-region peptides on maps of antibody light chains indicated that only a few different molecular species were present. This agrees with the observation of limited electrophoretic heterogeneity.

Modification of Lysines. Fully reduced and alkylated b_4 light chains were modified under various conditions. The percentage of lysines modified is shown in Table I. Complete blocking was not achieved either by tetrafluorosuccinylation or trifluoroacetylation. If maleylation were carried out in borate buffer alone, the protein was initially partially insoluble, yielding a cloudy solution. Although the solution cleared as the reaction progressed, 100% modification was not achieved. In order to solubilize the protein from the start, it was dissolved in freshly deionized 10 M urea; the urea sol-

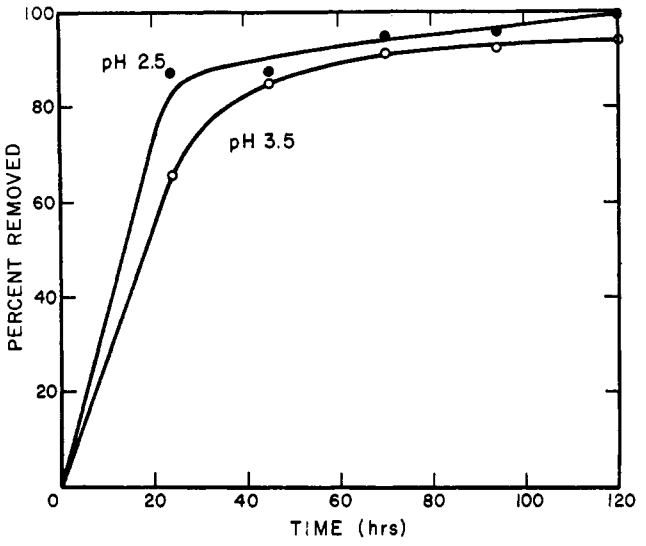

FIGURE 3: Rate of removal of maleyl groups. Completely maleylated light chains were incubated at 40° in 10% acetic acid, 5 M guanidine-HCl (pH 2.5) (●) or 10% acetic acid–1% pyridine–5 M guanidine-HCl (pH 3.5) (○). Aliquots were withdrawn at 24-hr intervals and analyzed for the number of lysines remaining modified by the DNP method.

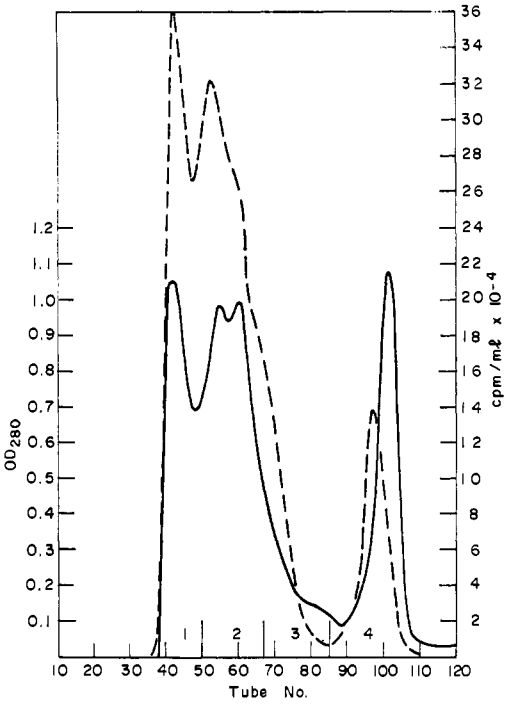

FIGURE 4: Elution of 130 mg of tryptic digest of completely maleylated, fully reduced, and alkylated 90 light chains from a 2.5 × 184 cm column of G-50 Sephadex in 6 M urea–0.1 M acetic acid. The cysteines involved in intrachain disulfide bonds were labeled with [^{14}C]iodoacetic acid. Fractions were pooled as indicated. (—) OD_{280} and (- - -) ^{14}C.

lution was then diluted fivefold with borate buffer. Subsequent addition of maleic anhydride produced complete maleylation within the sensitivity of the DNP assay method. The hydroxylamine–$FeCl_3$ test indicated that 10–20% of the hydroxyamino acids were esterified; no evidence was found for esterification of phenolic hydroxyls.

Demaleylation. The removal of maleyl groups by incubation in acidic guanidine solutions is shown in Figure 3. Guanidine was necessary to keep the maleylated protein in solution; without guanidine, it precipitated and little demaleylation occurred.

During the initial phase of hydrolysis, the half-life of the maleylamino group was about 12 hr at pH 2.5 and 19 hr at pH 3.5. A small proportion of the maleyl groups were removed only with difficulty. At pH 3.5 some lysines remained blocked even after 120-hr incubation. Incubation at pH 2.5 for 120 hr was adopted as the method of demaleylation to be used.

Peptide maps of demaleylated and umodified light chains showed no observable differences while few peptides in maps of maleylated light chains were found in the same position as peptides from untreated samples. Radioautographs confirmed these findings. This indicates that the deblocking procedure did not alter the polypeptide chain to any great extent. It also provides evidence that if hydroxyamino acids were maleylated, the esters were hydrolyzed during demaleylation.

As a further check that demaleylation did not break peptide bonds, a Bence Jones protein (κ 26, kindly supplied by Dr. Corrado Baglioni) was maleylated, demaleylated and subjected to two cycles of Edman degradation in the automatic sequenator (Waterfield *et al.*, 1970). The amino-terminal aspartic acid and second-residue isoleucine were obtained in fair yield (30%) with no more background than usual. Peptide-bond cleavages during demaleylation would be reflected by a high background or the appearance of additional amino termini in this type of analysis.

Fragments from a Tryptic Digest of Maleylated 90 Light Chain. Light chain 90 (130 mg) was fully reduced and alkylated with [^{14}C]iodoacetic acid, completely maleylated, and digested with trypsin. The elution pattern of this digest from a 2.5 × 184 cm column of G-50 Sephadex in 6 M urea–0.1 M acetic acid is shown in Figure 4. The total recovery of material from the column was greater than 90%. Little material eluted in the position of intact light chain indicating that the tryptic digestion had gone essentially to completion. Most of the material was found in two major fractions. Fraction 1 contained 25% of the starting material and had a molecular weight, estimated from previous calibration of the column, of about 16,000. Fraction 2 accounted for 45% of the starting material and had an estimated molecular weight of 5000–7000. Fraction 3 with a molecular weight of about 3500 contained about 15% of the material. The ^{14}C activity eluting near the end of the column probably reflected residual di-*S*-carboxymethyldithiothreitol which was not removed during dialysis in acetylated membranes. The optical density in this region was primarily caused by residual maleic acid.

The amino acid compositions of the G-50 fractions expressed as mole percent are given in Table II. The composition of the intact light chains is included for comparison. Fraction 1 contained a low level of methionine while fractions 2 and 3 contained substantial amounts of this residue. In rabbit light chains, methionine has so far been found only within the first five positions from the amino terminus (Jaton *et al.*, 1971; Hood *et al.*, 1970). The presence of methionine in fractions 2 and 3 therefore suggested that these contained amino-terminal peptides while the absence of methionine from fraction 1 indicated that it originated from some other region. Fraction 4 appeared to contain a mixture of small peptides. The expected carboxyl-terminal peptide, Gly-Asx-SCMCys (Appella *et al.*, 1970; Frangione and Lamm, 1970),

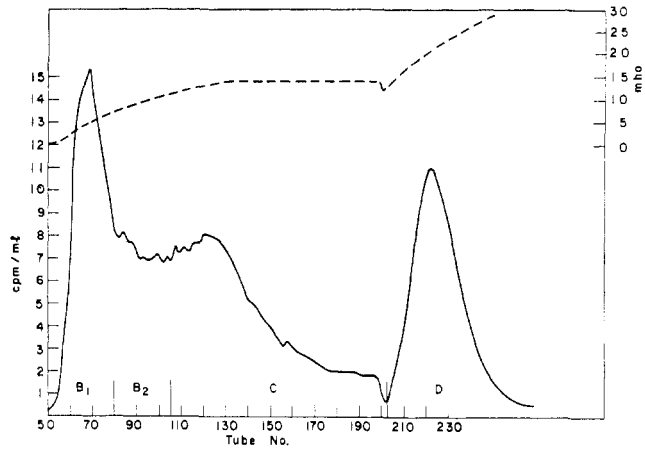

FIGURE 5: DEAE-Sephadex A-25 chromatography of fraction 2. Demaleylated fraction 2 (50 mg) was dissolved in 5 ml of 0.005 M NH_4HCO_3; the pH was adjusted to 8.5 and the conductivity to 0.46 mho. The column was washed with 100 ml of the same buffer. The column was first eluted with a 1000-ml convex gradient of NH_4HCO_3 (pH 8.5) from 0.005 to 0.4 M. The second convex gradient was from 0.3 to 1.0 M. Fractions were pooled as indicated. (—) ^{14}C, (---) conductivity.

was probably present in highest concentration since these amino acids were the most prevalent.

Fraction 4. After demaleylation and desalting, fraction 4 was subjected to high-voltage paper electrophoresis at pH 6.5. Previous experiments with fraction 4 from tryptic digests of maleylated pool light chains which had been carboxymethylated with [^{3}H]iodoacetic acid after mild reduction indicated that the carboxyl-terminal tripeptide migrated with a dinitrophenyl-*S*-carboxymethylcysteine (DNP-SCMCys) marker. Material in other portions of the electrophoretogram contained no SCMCys. When fraction 4 from the antibody preparation was subjected to high-voltage paper electrophoresis, the material which migrated with DNP-SCMCys contained only the expected amino acids: Gly, Asx, SCMCys. The other areas of the paper did not contain sufficient material for characterization.

TABLE II: Amino Acid Compositions of Antibody G-50 Fractions.

	Mole %				
	Light Chain	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Lys	4.05	3.19	3.68	3.60	4.28
His	0.58	0.88	0.41	0.33	0.72
Arg	1.27	0.41	0.61	2.92	4.38
SCMCys	3.37	3.09	2.39	2.05	8.21
Asx	8.78	11.29	8.70	7.00	25.53
Thr	13.07	15.31	13.10	8.40	3.58
Ser	10.00	8.98	10.03	11.76	7.71
Glx	9.92	8.95	10.43	9.21	5.79
Pro	5.64	5.50	6.67	9.33	3.96
Gly	8.43	8.47	8.65	8.78	14.59
Ala	7.68	6.62	7.28	9.89	5.69
Val	9.92	10.89	10.31	9.70	4.51
Met	0.66	0.05	0.76	1.11	0.79
Ile	3.13	2.87	3.32	4.33	2.64
Leu	4.68	4.27	4.98	6.35	4.87
Tyr	5.02	5.02	5.01	3.44	1.69
Phe	3.04	3.22	2.30	1.76	1.06
Trp	0.76	1.09	1.41	0.25	0.00

TABLE III: Amino Acid Compositions of DEAE Fractions.

	Residues/Peptide		
	2B	2C	2D
Lys	1.7	2.6	2.3
His	0.1	0.5	0.5
Arg	0.7	0.3	0.4
SCMCys	0.9	2.1	2.3
Asx	3.5	7.0	6.8
Thr	4.7	11.4	9.4
Ser	4.5	6.4	6.2
Glx	5.9	6.5	6.2
Pro	2.8	3.1	2.8
Gly	4.3	5.6	6.8
Ala	4.8	3.6	4.1
Val	5.8	6.4	5.6
Met	0.9	0.6	0.1
Ile	2.2	1.8	1.9
Leu	2.9	3.1	3.1
Tyr	1.6	3.7	3.9
Phe	0.5	1.8	2.2
Trp	0.3	0.7	0.6
Molecular weight	5000	7200	7000
Number of residues	48	67	65
Overall yield (%)	30	18	7

DEAE-Sephadex Chromatography of Fraction 2. After demaleylation and desalting, the major methionine-containing fraction (2) was subjected to ion-exchange chromatography on DEAE-Sephadex. Figure 5 shows the elution pattern obtained with 50 mg of fraction 2. About 85% of the starting material was recovered. Fraction 2B containing approximately 15% of the starting material appeared as soon as the gradient was started. This tailed into a second peak, fraction 2C, which eluted at 0.2 M NH_4HCO_3 and contained 29% of the material. The second stage of the gradient eluted a third fraction, 2D which accounted for 19% of the material. No further material eluted at salt concentrations up to 1 M.

Characterization of Fractions. The molecular weights of the major fractions were determined by gel filtration. Fractions 2C and 2D gave single symmetrical peaks which corresponded to molecular weights of 7200 and 7000, respectively. Approximately two-thirds of fraction 2B had a molecular weight of about 5000 while the rest was material with a molecular weight of around 3500. Fraction 1 after demaleylation produced two peaks containing roughly equal amounts of material with molecular weights of 10,000 and 6000. Little material eluted in the position of intact light chain.

The amino acid compositions of the DEAE fractions together with the final yields are given in Table III. These have been calculated as average compositions normalized to the molecular weights. The values obtained for SCMCys by amino acid analysis agreed closely with those calculated from the radioactivity. Difficulty in determination of arginine in

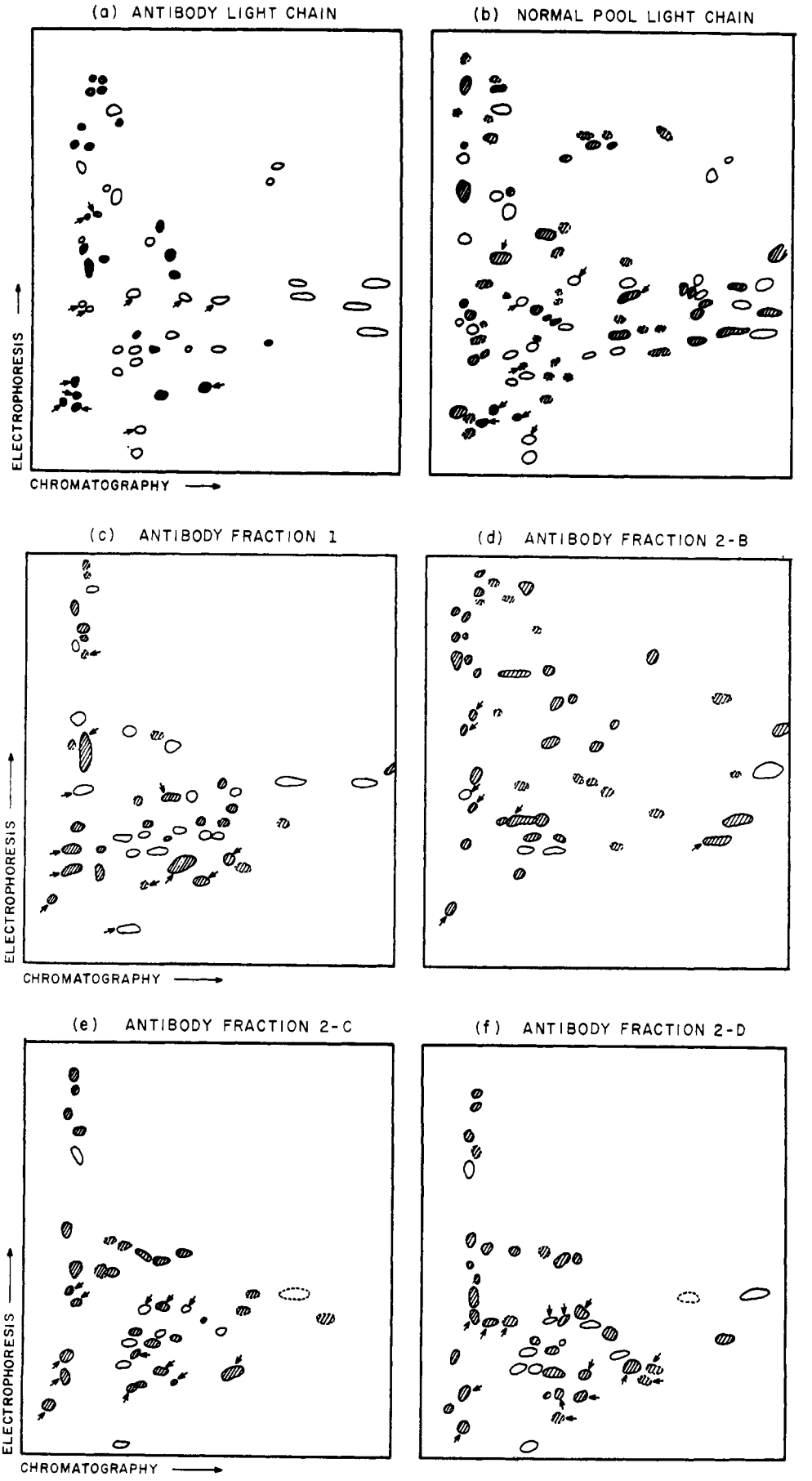

FIGURE 6: Schematic drawings of peptide maps of isolated fractions. 2 mg of each fraction was digested with chymotrypsin and mapped. For comparison maps of antibody and pool light chains which were maleylated, digested with trypsin, demaleylated, and digested with chymotrypsin have been included. Variable peptides are indicated by shading. Arrows denote radioactive peptides.

TABLE IV: Edman Degradations of Peptides.

Residue Positions from Amino Terminus	1	2	3	4	5	6	7	8
Intact light chain	Ala Asx	Val Phe Leu	Val Leu Ile Ala	Thr Val	Thr			
2B	Ala Asx	Val Phe Leu	Val Leu Ile Ala	Thr Val	Thr			
2C	Ser	Ala Tyr Thr	Phe	Pro	Asx	Val	Thr	
2D	Phe	Ser[a]	Gly	Ser[a]	Gly			
1	Ser Phe Pro	Val	Thr Pro Val	Pro	Thr	Val	Leu	Ile

[a] Ser inferred from Ala on amino acid analysis after HI hydrolysis and absence on direct gas–liquid chromatography of the PTH-amino acid.

hydrolysates was encountered throughout the work. Peptide hydrolysates produced low, broad arginine peaks which made integration, either manually or automatically, inaccurate. While fraction 2-B was quite different from the others, fraction 2C and 2D showed significant compositional differences only in threonine, glycine, alanine, valine, and methionine.

Chymotryptic peptide maps (Figure 6) of the individual fractions contained fewer peptides than did the maps of intact light chain. Comparison of the map of fraction 1 to that of whole light chain revealed that several peptides were missing from fraction 1. This fraction did contain many "constant-region" peptides in contrast to fraction 2B which contained only a few. Fraction 2C and 2D had very similar peptide maps containing several "constant-region" peptides as well as several variable peptides. These two fractions had nearly identical radioautographs which differed from those of fraction 1 and fraction 2B. The radioactive peptides in fraction 2B were quite different from those in any of the other fractions.

The similarity of the peptide maps and radioautographs of fractions 2C and 2D together with their comparable size and amino acid composition suggests that these two peptides originated from the same region of the light chain. Fraction 2B and fraction 1 are different from each other and from fractions 2C and 2D; this provides an indication that these represent three different parts of the light chain. The large number of "constant-region" peptides in fraction 1 suggests that this fraction contains at least part of the constant region while the lack of constant-region peptides in fraction 2B favors placing it in the variable region.

Amino-terminal sequences were determined by the manual Edman technique. Table IV lists the residues obtained at each step of the peptide degradations in the order of their prevalence.

With both the intact light chain and fraction 2B, two to four major alternatives were present at each step. Fraction 2B had exactly the same residues at each position as the intact light chain up through step five. After this point, the low yield and high background precluded identification of the residue removed. The other peptides had quite dissimilar amino-terminal sequences. Thus fraction 2B must contain the amino-terminal peptides.

Fraction 2D gave a fairly clear amino-terminal sequence. The sequence Phe-Ser-Gly-Ser-Gly has been found after an arginine at position 61 in virtually all κ chains examined through this region.

A single residue predominated at each position in fraction 2C except for position 2. This sequence could not be placed by homology with any of the published data, but M. D. Waterfield (1971, personal communication) has observed a similar sequence present in unfractionated mixtures of arginine peptides from rabbit light chains. A peptide with the sequence Tyr-Phe-Pro-Asx-Val-Thr has been isolated from a tryptic digest of an unblocked rabbit light chain (Fraser *et al.*, 1972).

The sequence of fraction 1 was heterogeneous in the beginning but the last five residues suggest that it belongs near the beginning of the constant region. Table V shows the homology of this sequence with fragments from two other rabbit light chains sequenced in this laboratory (A. D. Strosberg, J. D. Capra, and E. Haber, 1971, unpublished data) and the reported mouse and human κ sequences.

Discussion

A prerequisite for this approach is that the lysines be completely blocked. Neither tetrafluorosuccinylation nor trifluoroacetylation produced complete modification of light-chain lysines. Maleylation was more successful. If precautions were taken to insure that the protein was completely dissolved, complete modification was obtained. As suggested by the results of the hydroxylamine–$FeCl_3$ test, it is likely that some hydroxyamino acids were esterified under these conditions as has been found for lysozyme (Habeeb and Atassi, 1970) and tobacco mosaic virus (King and Perham, 1971). In this case sterification is not a serious disadvantage since the maleyl esters were removed during demaleylation. The observed lack of esterification of phenolic hydroxyls is expected because of the rapid rate of hydrolysis of these esters at neutral pH. King and Perham (1971) have shown that maleyl esters of tyrosine are completely destroyed in a few minutes at pH 8. In the present method, all such esters would be destroyed during the incubation after the addition of the anhydride.

Demaleylation required prolonged incubation at pH 2.5 but went essentially to completion. The half-lives of the maleylamino groups on light chains observed in this study are slightly longer than those reported by Butler *et al.* (1969). For ϵ-maleyllysine they reported a half-life of about 9 hr at pH 2.5 and 11–12 hr at pH 3.5; in light chains the half-life of the maleyl group appeared to be about 12 hr at pH 2.5 and 19 hr at pH 3.5.

The demaleylation procedure did not appear to cause other significant changes in the light-chain structure. An increase in α amino groups after demaleylation of a Bence Jones protein was not found by the Edman technique. Peptide maps of demaleylated light chains or their radioautographs were

TABLE V: "Switch" Region Peptide Sequences.

Position	108	109	110	111	112	113	114	115	116	117	118
Human	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe
Mouse	Arg	Ala	Asp	Ala	Ala	Pro	Thr	Val	Ser	Ile	Phe
Rabbit 7[a]	Gly	Asp	Pro	Val	Ala	Pro	Thr	Val	Leu	Ile	Phe
Fraction 1 (rabbit 90)			Ser	Val	Thr	Pro	Thr	Val	Leu	Ile	
			Phe		Pro						
			Pro		Val						

[a] Unpublished data of A. D. Strosberg, J. D. Capra, and E. Haber.

not significantly different from those of untreated light chains.

The tryptic digestion of maleylated light chains went essentially to completion as evidenced by the lack of appreciable material eluting in the light-chain position during G-50 chromatography, by the low level of methionine present in fraction 1, and by the peptide map of fraction 1, which differed from the intact light chain. The small number of peptides produced by tryptic digestion indicate that maleylation was complete. The recovery of material from both the G-50 and DEAE-Sephadex columns was sufficiently high to make it probable that no major peptides were lost.

Fraction 2B appeared to contain the amino-terminal peptides. Most of the peptides contained in this fraction were about 48 residues in length and had a molecular weight slightly less than one-fourth that of whole light chain. Peptide maps indicated that several constant-region peptides were missing, and the amino acid composition differed from that of whole light chain. The amino-terminal sequence of this fraction was identical with that of the light-chain preparation from which it was derived. The appearance of up to four residues at each position of the intact light chain reflects the presence of four sequences, in agreement with the observation of four electrophoretic species by disc gel electrophoresis. While two different sizes of peptides were observed by gel filtration of this fraction, both had the same amino-terminal sequence. This suggests different sites for tryptic cleavage, *i.e.*, different locations of the arginine.

Fraction 2C and 2D had very similar molecular weights, amino acid compositions, and peptide maps. The amino-terminal sequences, however, were quite different. These peptides probably include homologous regions of different light chains. The amino-terminal sequence of 2D, Phe-Ser-Gly-Ser-Gly, indicates that this peptide arose from tryptic cleavage at an arginine in a position homologous to position 61. This sequence has been found in practically all κ chains and the first four residues occur in most λ chains. Its conservation during the evolution of mouse and human light chains predicts its presence in rabbit light chains.

Fraction 2C was slightly larger than fraction 2D and had an entirely different amino-terminal sequence which, unfortunately, could not be placed by homology. It is unlikely that 2C arose from a cleavage on the amino-terminal side of position 61 because arginine has been found at this position in every light chain sequenced. Fraction 2C might start on the carboxyl-terminal side of position 66 and extend further toward the carboxyl-terminal end of the chain than 2D.

The peptide maps of fraction 2C and 2D contained several constant-region peptides suggesting that they may extend into the constant region. It must be remembered, however, that some constant region peptides may originate from conserved stretches in the amino-terminal or variable half of the light chain. The molecular weight of fraction 2D, which appears to start at position 62, indicates that it does extend past residue 107 into the true constant region. Since fraction 2C appears to contain most of the same peptides as 2D, it may also extend into the constant region.

The amino-terminal sequence data on fraction 1 indicate that one of the component peptides begins near the start of the constant region. By homology the first residue is placed at position 110 which is probably adjacent to an aspartic acid at position 109. Thus the fragment appears to have originated from a cleavage at aspartic acid. Selective clevage of aspartyl peptide bonds is known to occur under mildly acidic conditions at elevated temperatures. Tsung and Fraenkel-Conrat (1965) found a $T_{1/2}$ of 5.5 hr for the cleavage of aspartyl peptide bonds at 105° in 0.03 M HCl. The rate of hydrolysis is markedly dependent on temperature (Schultz, 1967) and would be expected to be much lower at 40°, the highest temperature to which fraction 1 was subjected. Rate of hydrolysis is also dependent on the adjacent residue, aspartyl–proline bonds being particularly labile (Piskiewicz *et al.*, 1970).

Fraction 1 contained species of two different molecular weights. From the work of Appella *et al.* (1970), it was expected that the constant region would be obtained as one large peptide plus the carboxyl-terminal tripeptide. This predicts that the larger peptide (mol wt 10,000) originates from the constant region. The smaller peptide may be a variable-region peptide homologous to those in fractions 2C and 2D. Perhaps in some individual light chains there is no arginine near the beginning of the constant region. Tryptic digestion of these chains after maleylation would produce a peptide with a molecular weight 16,000 (Figure 7A). Cleavage at the Asp–Pro bond during demaleylation would result in the observed mixture of two peptides with molecular weights of 10,000 and 6000.

A combination of an amino-terminal peptide of 48 residues, peptide 2C with 67 residues, and a 10,000 molecular weight constant region (~96 residues) plus the carboxyl-terminal tripeptide would account for a whole light chain (Figure 7B). In the case of the smaller amino-terminal peptide or peptide 2D which starts at position 62, a small variable-region peptide of about 14 residues is missing (Figure 7C,D). This is probably located in fraction 3.

The SCMCys analyses also suggest that a small peptide is missing. Instead of the four cysteines expected in the variable region, a combination of 2B with 2C or 2D would only

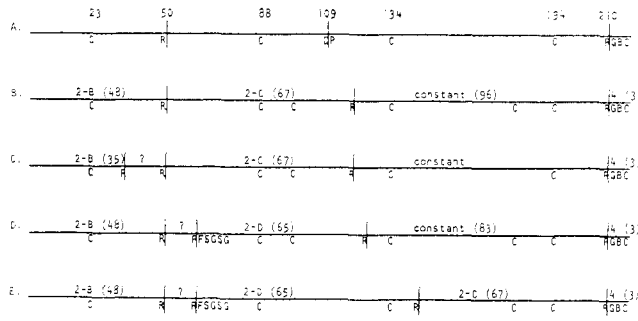

FIGURE 7. Hypothetical arrangements of peptides. The schematic diagrams present possible arrangements of the observed peptides in the whole light chain. The letters below the line indicate in the single letter code (Dayhoff, 1969) the amino acid presumed to occupy the position; above the line are the peptide designations with the numbers of residues in parentheses. Part A shows the production of two peptides from a larger one by Asp-Pro cleavage. Part B combines the larger amino-terminal peptide with a variable-region 2C, a 10,000 mol wt constant region, and the C-terminal tripeptide. Part C is a similar combination using the smaller amino-terminal peptide. Part D represents a chain with 2D in place of 2C. Part E pictures the possibility that 2C is a constant-region peptide from a chain including 2D and the larger amino-terminal peptide.

account for three. The present data suggest that one cysteine is present in the first 48 residues and that two occur after residue 66. This agrees with the retention of the two very conservative cysteines at positions 23 and 88. The location of an extra cysteine after position 66 is also in agreement with E. Appella's (1971, personal communication) recent scheme. He has proposed that the fourth cysteine occurs somewhere between residues 39 and 55. Our data suggest that it is located after position 48. Another possibility is that one of the extra cysteines is in the constant region. A cysteine-containing peptide recently sequenced in this laboratory (Fraser *et al.*, 1972) has been tentatively placed by homology as coming from the region around position 171 in the constant region. In this case (Figure 7E) peptide 2C may represent a carboxyl-terminal portion of the constant region and be combined with peptide 2D and the amino-terminal peptide 2B. A small variable-region peptide would be missing.

The amino-terminal sequences of the intact light chain were identical with the sequences of the amino-terminal peptides which emerged together during the purification, although they were separated from non-amino-terminal peptides. This suggests that the method should be generally applicable to isolating the amino-terminal peptides from the light chains of any rabbit antibody. The similarities between the chromatographic behavior of pool and antibody material provide an indication that the method will also be useful for isolating homologous internal variable-region peptides.

The methods evolved in this investigation facilitate the sequencing of large peptides from identifiable regions of antibody light chains. They should aid in establishing the principles of variability of the heterogeneous group of molecules which comprise antibodies.

Acknowledgment

The authors thank Dr. Jean-Claude Jaton for his help in initiating this study. We deeply appreciate the skillful technical assistance of Miss Clare Corbett and Mr. John Wechsler.

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