Gennari, G., Cauzzo, G., & Jori, G. (1974) *Photochem. Photobiol. 20*, 497–499.
Girotti, A. W. (1975) *Biochemistry 14*, 3377–3383.
Girotti, A. W. (1976a) *Biochem. Biophys. Res. Commun. 72*, 1367–1374.
Girotti, A. W. (1976b) *Photochem. Photobiol. 24*, 525–532.
Girotti, A. W. (1978) *J. Biol. Chem. 253*, 7186–7193.
Goldstein, B. D., & Harber, L. C. (1972) *J. Clin. Invest. 51*, 892–901.
Hsu, J., Goldstein, B. D., & Harber, L. C. (1971) *Photochem. Photobiol. 13*, 67–77.
Leibowitz, M. E., & Johnson, M. C. (1971) *J. Lipid Res. 12*, 662–670.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mauk, M. R., & Girotti, A. W. (1973) *Biochemistry 12*, 3187–3193.
Pryor, W. A., Stanley, J. P., & Blair, E. (1976) *Lipids 11*, 370–379.
Ralston, G., Dunbar, J., & White, M. (1977) *Biochim. Biophys. Acta 491*, 345–348.
Ray, W. J., & Koshland, D. E. (1960) *Brookhaven Symp. Biol. 13*, 135–150.
Rega, A. F., Weed, R. I., Reed, C. F., Berg, G. G., & Rothstein, A. (1967) *Biochim. Biophys. Acta 147*, 297–312.
Rosenberg, S. A., & Guidotti, G. (1968) *J. Biol. Chem. 243*, 1985–1992.
Roubal, W. T., & Tappel, A. L. (1966) *Arch. Biochem. Biophys. 113*, 150–155.
Schothorst, A. A., van Steveninck, J., Went, L. N., & Suurmond, D. (1970) *Clin. Chim. Acta 28*, 41–49.
Shimasaki, H., & Privett, O. S. (1975) *Arch. Biochem. Biophys. 169*, 506–512.
Spikes, J. D., & MacKnight, M. L. (1970) *Ann. N.Y. Acad. Sci. 171*, 149–162.
Steck, T. L. (1972) *J. Mol. Biol. 66*, 295–305.
Steck, T. L., & Fox, C. F. (1972) in *Membrane Molecular Biology* (Fox, C. F., & Keith, A. D., Eds.) pp 27–75, Sinauer Associates, Stamford, CT.
Steck, T. L., & Kant, J. A. (1973) *Methods Enzymol. 31*, 172–180.
Steck, T. L. & Yu, J. (1973) *J. Supramol. Struct. 1*, 220–232.
Stocks, J., & Dormandy, T. L. (1971) *Br. J. Haematol. 20*, 95–111.
Straight, R., & Spikes, J. D. (1978) *Photochem. Photobiol. 27*, 565–569.
Tappel, A. L. (1975) in *Pathobiology of Cell Membranes* (Trump, B. F., & Arstila, A. U., Eds.) pp 145–170, Academic Press, New York.
Thomas, M. J., & Foote, C. S. (1978) *Photochem. Photobiol. 28*, 403–408.
Tomita, M., Irie, M., & Ukita, T. (1969) *Biochemistry 8*, 5149–5160.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005–8018.
Wills, E. D. (1964) *Biochim. Biophys. Acta 84*, 475–477.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176–9184.

Isolation and Characterization of an Active Variable Domain from a Homogeneous Rabbit Antibody Light Chain†

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ABSTRACT: The variable domain (V_L) of allotype b4 light chains of rabbit IgG was isolated from both nonimmune heterogeneous IgG and a homogeneous antibody directed against type III pneumococcal polysaccharide. Light chains were first isolated and then cleaved under mild acidic conditions between residues 109 and 110. Reduction with dithiothreitol in guanidine hydrochloride cleaved both intradomain disulfide bridges as well as the interdomain disulfide bridge joining the variable and constant domain. The sulfhydryl groups were protected after reduction by *p*-chloromercuribenzoate. V_L was isolated from this mixture of variable and constant domains by affinity chromatography, utilizing sheep antibodies directed against a peptide including residues 110–211 from nonimmune IgG light chain. The isolated V_L domain was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and automated Edman degradation. V_L from a homogeneous antibody was treated with dithiothreitol to remove *p*-chloromercuribenzoate, reoxidized, and recombined with homologous heavy chain. The binding of this recombinant to type III pneumococcal polysaccharide was identical with that of the light-chain–heavy-chain recombinant.

Immunoglobulin polypeptide chains are composed of linear repeating regions of homologous sequence, ~110–120 amino acid residues in length (Edelman, 1970). Sequence variability is located in the amino-terminal one-quarter of the heavy chain and the amino-terminal one-half of the light chain, suggesting that these regions constitute the antibody-combining site (Kabat, 1967). Their participation in antigen binding has been confirmed directly by X-ray crystallography (Segal et al., 1974; Poljak et al., 1973). X-ray studies have also supported the hypothesis that each homology region is an independent domain that has a tightly folded structure stabilized by a

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disulfide bond. The isolation of a fully active hapten-binding fragment (Fv)[1] consisting of only the variable region of the heavy chain (V_H) and the variable region of the light chain (V_L) from the DNP-binding murine myelomas MOPC 315 and XRPC-25 (Hochman et al., 1973; Sharon & Givol, 1976) has further verified the domain theory.

Immunoglobulin variable domains have been isolated from heterogeneous IgG and myeloma proteins for which no corresponding hapten or antigen is known (Smith & Dorrington, 1972; Karlsson, 1971, Lin & Putnam, 1978; Mole et al., 1975; Michaelson et al., 1977; Rodwell & Karush, 1978). Most of these studies employed enzymatic cleavage in which the desired immunoglobulin domain is readily isolated by molecular sieve chromatography or is the only nondialyzable fragment remaining. Constant region domains have also been isolated [reviewed by Winkelhake (1978)]. The results indicate that, in general, isolated immunoglobulin domains retain the same or similar tertiary structure which they assume as part of the intact immunoglobulin. The domains are still capable of interacting with other immunoglobulin chains (Smith & Dorrington, 1972; Karlsson, 1971; Lin & Putnam, 1978), and they retain tertiary structure dependent properties such as the allotype (Mole et al., 1975).

The hapten-binding experiments on myeloma fragments utilize a small hydrophobic molecule which requires only a portion of the antigen-binding cavity. The DNP hapten is bound by a significant percentage of antigen-binding myeloma proteins. The contribution of the constant domains to the binding of larger antigens remains unclear. It is important to extend these results to the binding of an antigen by the variable domain of an elicited homogeneous antibody. Two methods employing enzymatic cleavage have been reported for the production of rabbit antibody V_H (Mole et al., 1975; Rosemblatt & Haber, 1978). The V_H from a homogeneous antibody shows a partial regaining of binding activity upon recombination with light chain (Rosemblatt & Haber, 1978). A method for producing an active V_L, however, has been lacking. Under mild acidic conditions, the light-chain switch region is cleaved in high yield between aspartic acid at position 109 and proline at position 110 (Fraser et al., 1972). The acid-cleaved light chain, upon recombination with the homologous heavy chain, restores full antigen binding (Poulsen et al., 1972). However, as rabbit κ chains contain, in addition to the intradomain disulfide bonds, an extra disulfide bridge spanning the variable and constant domains (Strosberg et al., 1972, 1975), these two regions in the acid-cleaved light chain remained associated. In order to isolate V_L from C_L, the interdomain disulfide bridge must be reduced and the domains then separated.

In this study, the separation of V_L from C_L has been effected by the development of an immunoadsorbent containing sheep antibodies directed against a constant region peptide including residues 110–211. By use of a reversible cysteine-protecting group, the isolated V_L was reoxidized and recombined with the homologous heavy chain to yield an active antibody fragment.

[1] Abbreviations used: Fv, Ig fragment composed of the variable region of light chain and the variable region of heavy chain; V_H, variable region of Ig heavy chain; V_L, variable region of Ig light chain; DNP, 2,4-dinitrophenyl; C_L, constant region of light chain; Pth, phenylthiohydantoin; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoate; GAB buffer, guanidine–acetate buffer [5 M guanidine hydrochloride (Gdn·HCl) and 0.1 M sodium acetate, pH 5.5]; PBS, phosphate-buffered saline (0.005 M potassium phosphate, 0.15 M NaCl, and 0.02% NaN_3, pH 7.0); S3, type III pneumococcal polysaccharide.

Materials and Methods

General. Protein concentrations were determined by amino acid analysis following 24 h of hydrolysis at 110 °C in constant-boiling HCl in sealed evacuated tubes. Analyses were performed on a Durrum D-500 amino acid analyzer.

The method of Niall & Potts (1970) was used for manual Edman degradation. Automated Edman degradation was performed in a modified Beckman 890B sequencer using a 0.1 M Quadrol program described previously (Brauer et al., 1975). Phenylthiohydantoin (Pth) amino acids were identified and quantified by gas–liquid chromatography and high-pressure liquid chromatography (Margolies & Brauer, 1978). Thin-layer chromatography on polyamide sheets was also used (Summers et al., 1973).

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed according to Swank & Munkres (1971).

Light-chain allotypes were determined by Ouchterlony analysis or by radioimmunoassay (Gottlieb et al., 1975). Antiallotypic antisera were the gift of Dr. Charles Todd and Dr. Meredith Mudgett-Hunter.

Preparation of Light Chains and Acid Cleavage. The homogeneous antitype III pneumococcal polysaccharide antibody 3T74 was purified by affinity chromatography as described previously (Ehrlich et al., 1978; Cheng et al., 1973). Pooled allotype b4 IgG was isolated from nonimmune serum of New Zealand white rabbits by ion-exchange chromatography on columns of diethylaminoethylcellulose (DE-52 Whatman). Ig was eluted by using 0.01 M potassium phosphate and 0.02% sodium azide (pH 7.5).

Antibody or heterogeneous IgG was reduced and alkylated, and heavy and light chains were separated by a modification described previously (Ehrlich et al., 1978).

Light chains were subjected to acid cleavage under mild conditions as described by Poulsen et al. (1972). The reaction was carried out for 96 h to maximize the yield of cleavage between the V_L (1–109) and C_L (110–214) regions.

Preparation of Protected Cleaved Light Chains. Acid-cleaved light chains (29 mg) were dialyzed against 7 M Gdn·HCl and 0.5 M Tris-HCl, pH 8.2 (10 mg/mL), and then reduced for 90 min with 0.02 M dithiothreitol (DTT) added as a solid (Aldrich Chemical Co.) at 37 °C under nitrogen. To this solution was added an equal volume (3 mL) of 7 M Gdn·HCl, 0.5 M Tris-HCl, and 0.05 M *p*-chloromercuribenzoate (PCMB) (ICN Pharmaceuticals), pH 8.2. The mixture was incubated for 1 h at 20 °C. The PCMB-treated cleaved light chains were then dialyzed against 5 M Gdn·HCl and 0.1 M sodium acetate, pH 5.5 (GAB buffer), for 16 h. As acid cleavage of the light chain for 96 h is usually 70–90% complete, cleaved and uncleaved light chains were separated by gel filtration on Sephadex G-75 equilibrated in GAB buffer. (In order to conserve material, we did not perform this step with the homogeneous light chain from antibody 3T74.)

Preparation of C_L Peptide. Complete reduction and radioalkylation of light chains, modification of the alkylated chains with citraconic anhydride, and tryptic digestion of the citraconylated chains from nonimmune pooled b4 light chains were performed according to Cannon et al. (1978). Tryptic peptides from the citraconylated, fully reduced, and radioalkylated light chains were fractionated by Sephadex G-75 gel filtration and freed from salt as previously described (Cannon et al., 1978). The largest peptide obtained (residues 62–211) was acid cleaved (Poulsen et al., 1972) for 96 h and the reaction mixture applied to a 2.5 × 100 cm column of Sephadex G-75 equilibrated in GAB buffer. Fractions of 2.7

mL were collected and monitored for absorbance at 280 nm, and radioactivity was monitored by counting an aliquot (10 μL) in Bray's solution. Major peaks (including the C_L peptide 110–211) were pooled, freed from salt by dialysis in 3500 molecular weight cutoff tubing against 10% acetic acid, and lyophilized. The conditions used for the acid cleavage were sufficient to remove simultaneously the citraconyl groups from the peptides.

Preparation of C_L Peptide–Sepharose. The C_L peptide (residues 110–211) (5 mg) was dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl, and 3.5 M Gdn·HCl, pH 8.0, and reacted for 2 h at 20 °C with 2 mL of cyanogen bromide activated Sepharose 4B (Pharmacia).

Preparation of Sheep Antibodies Specific for Rabbit Light-Chain Constant Region. Light chains (32 mg) from pooled nonimmune b4 rabbit Ig were completely reduced and alkylated with iodoacetic acid as previously described (Cannon et al., 1978). Following acid cleavage for 96 h, the mixture was applied to a 20 mm × 125 cm Sephadex G-75 column equilibrated in 6 M urea and 1 N acetic acid in order to separate cleaved from uncleaved chains (Poulsen et al., 1972). The fractions containing cleaved light chains ($V_L + C_L$) were pooled and freed from salt on a Sephadex G-10 column, 3.5 × 100 cm, equilibrated in 0.03 N NH_4OH. The peptide mixture was lyophilized and stored at −20 °C.

Cleaved, reduced, and carboxymethylated light chains (20 mg) were suspended in 15 mL of 0.1 M sodium phosphate buffer, pH 7.0. To this suspension was added 75 μL of 50% glutaraldehyde (Fischer) dropwise. The suspension (1.25% in glutaraldehyde) was stirred for 1 h at 20 °C. Glutaraldehyde cross-linking of the antigen was stopped by the addition of 4 mL of 2.5 M lysine. The reaction mixture was dialyzed repeatedly against water in acetylated membranes, followed by lyophilization.

Seventy-kilogram Shropshire sheep were immunized intramuscularly monthly for 4 months with 5 mg of glutaraldehyde cross-linked cleaved light chains with complete Freund's adjuvant. Antisera collected weekly were tested for antibody production by the ring test using fully reduced and alkylated nonimmune b4 light chains as the antigen.

Sheep antiserum (3–6 mL) containing antibodies reacting with completely reduced and alkylated b4 light chains were applied to the C_L peptide–Sepharose immunoadsorbent column described above in order to isolate a fraction specific for the reduced and alkylated constant region. After being washed with PBS, the column was eluted successively with 2 M Gdn·HCl (12 mL) and then with 5 M Gdn·HCl (8 mL).

Preparation of Sheep Antirabbit C_L Immunoadsorbent. The fraction eluted with 5 M Gdn·HCl was diluted to a protein concentration of <0.5 mg/mL and dialyzed overnight against 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.0, at 4 °C. Precipitate was removed by centrifugation. The soluble antibodies (16 mg) were then reacted with 5 mL of CNBr-activated Sepharose 4B to make the specific anti-C_L (110–211) immunoadsorbent.

Purification of V_L. Acid-cleaved PCMB-treated light chains were diluted with GAB buffer until the protein concentration was less than 0.1 mg/mL. This was followed by dialysis against 6 L of PBS at 4 °C for 16 h. Preliminary dilution of the chains was necessary prior to dialysis since the PCMB-treated cleaved light chains were poorly soluble in PBS. Following dialysis, the small amount of precipitate which did appear was removed by centrifugation.

The acid-cleaved PCMB light chains (1–1.5 mg, 0.08 mg/mL) were applied to the sheep antirabbit C_L peptide immunoadsorbent (4 mL) at 4 °C which had been equilibrated in PBS. The column was eluted first with PBS until no further absorbance at 280 nm could be detected. The column was then washed with GAB buffer (20 mL) to remove bound protein. Thereafter, the column was reequilibrated with PBS (10 mL). The protein fraction which had been eluted with Gdn·HCl was dialyzed extensively against distilled water and lyophilized prior to amino acid analysis and automated Edman degradation, as was an aliquot of the unbound fraction. The remainder of the unbound fraction (V_L) was used for activity measurements.

Reoxidation of V_L. The unbound fraction of cleaved PCMB light chains from the sheep antirabbit C_L immunoadsorbent was dialyzed against 7 M Gdn·HCl and 0.5 M Tris-HCl, pH 8.2, for 16 h. The protein was reduced with 0.02 M DTT for 90 min at 37 °C under nitrogen. Thereafter, a solution containing 7 M Gdn·HCl, 0.5 M Tris-HCl, and 0.1 M mercaptoethanol was added to lower the protein concentration to less than 30 μg/mL, if necessary. The peptides were then dialyzed against 0.1 M Tris-HCl and 0.1 M mercaptoethanol, pH 8.0, at 20 °C without stirring for 6.5 h. The dialysis bag was transferred to a large volume (6 L) of 0.1 M Tris–acetate and 2 mM mercaptoethanol at 5 °C (pH 7.8 measured at room temperature) and dialyzed with stirring for 48 h with one change of dialysate. Since the isolated V_L domain contains three cysteine residues (Strosberg et al., 1975; Margolies et al., 1975), alkylation of the cysteine remaining after re-formation of the intradomain disulfide bridge was performed. The peptide was removed from the dialysis bag and incubated with 2.4 mM iodoacetamide for 20 min at 0 °C. After dialysis overnight against 0.01 M sodium acetate, pH 5.5, at 5 °C, the peptide was concentrated with Aquacide (Calbiochem) to 8 μg/mL and again dialyzed against 0.01 M sodium acetate and 0.02% sodium azide, pH 5.5, and stored at −20 °C.

Assay of Recombined Antibody Fragments for Binding Activity. The radiobinding assay for type III pneumococcal polysaccharide (S3) antibodies was analogous to the assay described by Chen et al. (1976) and has been detailed previously (Ehrlich et al., 1978). The activity of 3T74 light chains or putative 3T74 V_L was assayed by adding increasing amounts in 0.01 M sodium acetate, pH 5.5, and 0.02% sodium azide to a constant amount of 3T74 heavy chain in the same buffer. The recombined antibody or antibody fragments were added to [^{125}I]-S3-soybean trypsin inhibitor (Ehrlich et al., 1978). The antigen–antibody complex was precipitated in the presence of carrier nonimmune rabbit IgG by goat antirabbit IgG antiserum. The maximum percent binding of the [^{125}]-S3-soybean trypsin inhibitor was 50–65% depending on the antigen preparation.

Results

Isolation of C_L Peptide (110–211) from Nonimmune Rabbit Light Chains. Forty milligrams of fully reduced and alkylated, pooled nonimmune b4 light chains was citraconylated and subjected to tryptic digestion. The digest was fractionated by Sephadex G-75 gel filtration. The profile obtained was similar to that previously reported (Cannon et al., 1978). As the rabbit κ b4 light chain contains only one invariant arginine residue in the C region at position 211 (Chen et al., 1974) and one invariant arginine at position 61 in the variable region (Margolies et al., 1975), a large fragment including residues 62–211 may be isolated from this digest (see Figure 1). Additional arginine residues located amino-terminal to position 61 in heterogeneous nonimmune light chains resulted in smaller fragments that did not interfere with purification of the 62–211 peptide, which was obtained in 64% yield. In order to be certain that the peptide including residues 62–211 had not

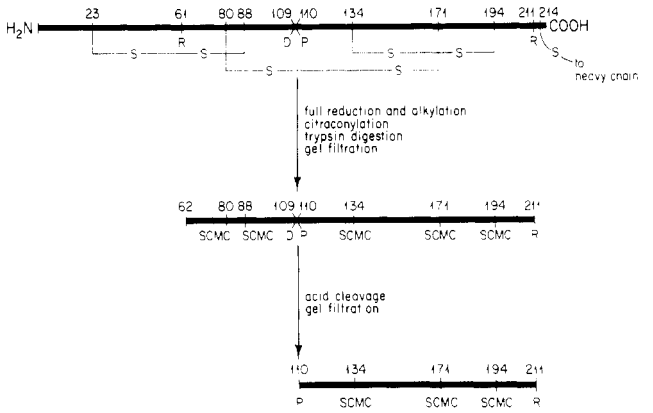

FIGURE 1: Structure of rabbit antibody b4 κ light chains, demonstrating the V_L intradomain disulfide bridge (23–88), the C_L intradomain disulfide bridge (134–194), and the interdomain disulfide bridge (80–171) (Strosberg et al., 1972, 1975; Margolies et al., 1975). The light chain may be cleaved under mild acidic conditions between aspartic acid at position 109 and proline at position 110 (Poulsen et al., 1972; Fraser et al., 1972). Invariant arginines are located at position 61 in the V_L and at position 211 in the C_L (Margolies et al., 1975; Chen et al., 1974). Following full reduction and alkylation, the citraconylated chains were digested with trypsin. The largest arginine peptide obtained spans residues 62–211. This peptide may be further cleaved in the switch region under mild acidic conditions to yield a peptide (110–211) including most of the constant region.

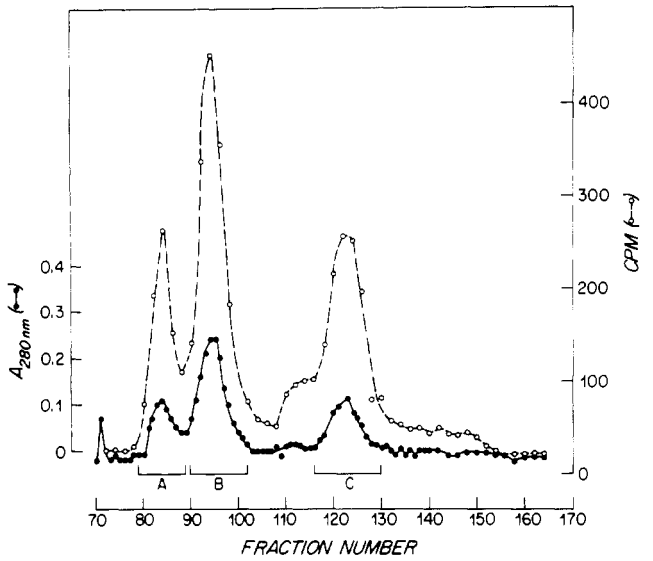

FIGURE 2: Isolation of C_L peptide (110–211) from nonimmune pooled b4 light chains. Light chains were fully reduced, alkylated, citraconylated, and digested with trypsin. The peptide spanning residues 62–211 was purified by gel filtration on Sephadex G-75 and cleaved in acid (see Figure 1). The acid-cleaved 62–211 peptide was then applied to a 2.5 × 100 cm column of Sephadex G-75 equilibrated in GAB buffer. Fractions contained in peak B were pooled and contained the C_L peptide (110–211).

undergone internal tryptic digestion on account of premature removal of citraconyl groups (Cannon et al., 1978), we carried out manual Edman degradation for three cycles on 31 nmol of peptide. A single sequence, Phe-Thr-Gly, was obtained. The yield of Pth-phenylalanine in the first cycle was 28 nmol. This sequence is identical with that beginning at position 62 in pooled b4 light chains (Strosberg et al., 1972) and light chains from homogeneous antibodies (Margolies et al., 1975). The remainder of the sample (30 mg) was subjected to mild acidic conditions known to cleave between residue 109, aspartic acid, and 110, proline (see Figures 1 and 2). The cleaved peptide was applied to a Sephadex G-75 column. The results of gel filtration are shown in Figure 2 and are consistent with

Table I: Amino Acid Analysis of C_L Peptide 110–211

residues	expected[a]	found[b]
Cys[c]	3	3.1
Asp	11	10.5
Thr	19	17.2
Ser	8	7.9
Glu	9	9.5
Pro	6	5.8
Gly	4	5.6
Ala	6	6.4
Val	12	11.6
Ile	3	2.9
Leu	4	4.1
Tyr	4	4.1
Phe	3	3.2
His	1	1.1
Lys	4	4.2
Arg	1	0.9
total	98	98.1

[a] Composition of b4 constant region from known sequence (Chen et al., 1974). [b] Average of four 24-h hydrolyses. Values are not corrected for hydrolytic losses. [c] Determined as *S*-(carboxymethyl)cysteine.

cleavage of the 62–211 peptide between residues 109 and 110 (Margolies et al., 1974). The major peak (B) was freed from salt and lyophilized. Amino acid analysis on an aliquot of this peptide is shown in Table I, where it is compared to the composition of the 110–211 peptide based on the known sequence. An aliquot (150 nmol) of the peptide from pool B was subjected to automated Edman degradation, revealing the following sequence: Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro. The yield of Pth-valine at cycle 2 was 110 nmol. The only evidence for contaminating sequences was that for the 62–211 peptide (Phe-Thr-Gly...), which was revealed by 6.5 nmol of Pth-phenylalanine at cycle 1. Thus, the 110–211 C_L peptide was contaminated with ~6% of a peptide containing a portion of the variable region. The yield of the C_L peptide (110–211) following acid cleavage of the 62–211 peptide was 70%. Pools A and C (Figure 2) correspond to the uncleaved 62–211 peptide and a peptide comprising residues 62–109, respectively, and were not examined further.

Preparation of Sheep Antirabbit C_L Immunoadsorbent. The purified C_L peptide (110–211) (5 mg) was coupled to CNBr-activated Sepharose. Sheep anticleaved b4 light chain antiserum (3–6 mL) was applied to this C_L peptide immunoadsorbent. Sheep antibodies specific for the C_L peptide (110–211) were eluted from the column with 5 M Gdn·HCl after a preliminary 2 M Gdn·HCl wash. The eluted antibodies (16 mg from three separate preparations) were dialyzed against 0.1 M sodium bicarbonate and 0.5 M sodium chloride, pH 8.0, at 4 °C and coupled to 5 mL of CNBr-activated Sepharose to make the sheep antirabbit C_L immunoadsorbent. The latter immunoadsorbent was used to purify V_L from PCMB-protected cleaved light chains.

Isolation and Characterization of V_L from Nonimmune Pooled Light Chains. Nonimmune b4 pooled light chains (29 mg) were acid cleaved and fully reduced, and the cysteine residues were protected with PCMB. Uncleaved light chains were separated from the cleaved light chains by gel filtration on Sephadex G-75. In Figure 3A $NaDodSO_4$–polyacrylamide gel analysis of the isolated cleaved light chains is shown which demonstrates that all the peptides in that fraction have a molecular weight of ~12 000, corresponding to the expected size for an isolated V_L and/or C_L domain.

An aliquot of the cleaved light chains was subjected to sequence analysis. The sequence was compared to that of the same preparation of light chains prior to modification by acid

Table II: Sequenator Analyses of Pooled Nonimmune b4 Light-Chain Fractions

fraction	Pth amino acids recovered at each cycle[a]						
	1	2	3	4	5	6	7
unmodified light chains	Ala (15.8) Asp (3.7) Val (3.3) Leu (1.9)	Val (13.9) Tyr (8.7) Asp (4.1)	Val (14.3) Leu (5.6) Met (1.3)	Met (10.6) Val (6.7) Thr (2.2)	Gln (7.6) Met (5.4) Thr (4.7)	Gln (7.4) Thr (3.7)	Thr (5.4) Gln
acid-cleaved[b] light chains	Ala (11.6) Asp (9.7) Val (1.9) Leu (1.1)	*Val* (23.0) Tyr (4.6) Asp (7.0)	Val (7.6) Leu (3.4) Asp (7.0)	Met (5.7) Val (3.3) Thr (4.1)	*Thr* (6.0) Gln (3.0) Met (1.7) Gly (1.3)	Gln (6.7) Thr (1.6)	Thr (1.1) Gln
	Pro (2.9)		*Ala* (14.1)	*Pro* (8.1)		*Val* (14.8)	*Leu* (14.4)
fraction of acid-cleaved light chains not bound to anti-C_L peptide immunoadsorbent	Ala (6.4) Asp (1.1) Val (1.1)	*Val* (3.4) Trp (3.6) Asp (1.2)	Val (1.9) Leu (1.5)	Met (3.6) Val (1.7) Thr (1.0)	Met (2.4) Gln (1.0) *Thr* (1.0)	Thr (2.6)	Thr (2.5)
fraction of acid-cleaved light chains binding to immunoadsorbent	Ala (7.2) Asp (1.7) Val (1.6)	Val (21.7) Asp (1.0)	Val (4.6) Leu (2.5) Met (1.7)	Met (2.8) Val (2.0) Thr (1.1)	Thr (6.9) Met (1.1)	Gln (1.7)	
	Pro (3.6)		*Ala* (12.9)	*Pro* (3.2)		*Val* (10.5)	*Leu* (10.8)
expected C_L sequence[c]	*Pro*	*Val*	*Ala*	*Pro*	*Thr*	*Val*	*Leu*

[a] Yields (nmol) of Pth amino acids based on gas–liquid chromatography and LC are given in parentheses. Amounts less than 1 nmol are omitted, except for the unbound fraction. [b] Following cleavage at the aspartyl-109–prolyl-110 peptide bond under mild acid conditions, light chains were fully reduced and cysteine residues were reacted with *p*-chloromercuribenzoate. Cleaved light chains ($V_L + C_L$) were separated from uncleaved light chains by gel filtration on Sephadex G-75; an aliquot of the cleaved light chains was subjected to Edman degradation. [c] Sequence of the rabbit κ allotype b4 light chains beginning at position 110 was reported previously (Fraser et al., 1972; Strosberg et al., 1972).

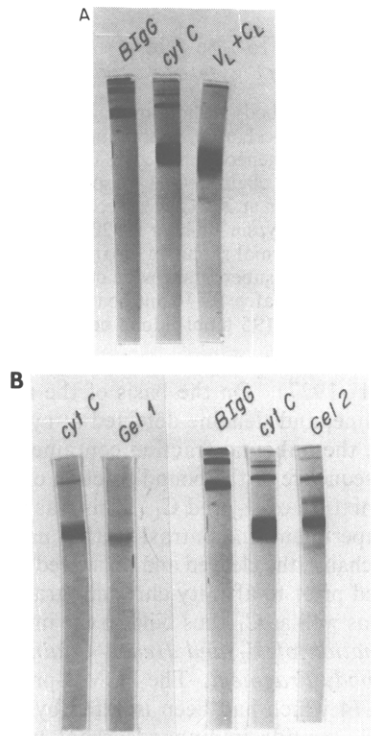

FIGURE 3: (A) $NaDodSO_4$–polycrylamide gels (12.5%) of a mixture of V_L and C_L from pooled nonimmune b4 L chains. Light chains were acid cleaved, fully reduced, PCMB-treated, and separated from uncleaved light chains. Molecular weight standards (bovine IgG and cytochrome *c*) are included for comparison. The approximate molecular weight of the mixture of V_L and C_L was 12000. (B) $NaDodSO_4$–polyacrylamide gels (12.5%) of V_L from homogeneous antibody 3T74. The 3T74 light chain was acid cleaved, reduced, PCMB-treated, and applied to the sheep anti-C_L immunoadsorbent. The unbound fraction contained V_L (gel 1) (see Table III). The fraction remaining bound to the column which was eluted with Gdn·HCl (gel 2) contained a mixture of V_L and C_L as well as a small amount of uncleaved light chain (see Table III).

cleavage (Table II). The amino acid sequence heterogeneity of pooled nonimmune rabbit light chains has been previously characterized (Hood et al., 1970; Jaton et al., 1971) and includes not only multiple amino acid substitutions but three different amino-terminal chain lengths (Cannon et al., 1976). Following acid cleavage, the amino acid sequence found resembles that of unmodified light chains except for an additional residue identified at cycles 1, 3, 4, 6, and 7, consistent with the known b4 constant region amino acid sequence beginning at position 110 (Pro-Val-Ala-Pro-Thr-Val-Leu...) (Strosberg et al., 1972; Fraser et al., 1972, Chen et al., 1974). (The amino acid substitutions at cycles 2 and 5, valine and threonine, respectively, are shared by both V_L and C_L sequences.) The sum of Pth amino acids ascribable to the V_L at each position is approximately equal to the amount of Pth amino acids arising from the C_L and is consistent with acid cleavage resulting in an equimolar mixture of V_L and C_L.

Following separation from the uncleaved light chains, the PCMB acid-cleaved light chains (1–1.5 mg) were applied to the sheep antirabbit C_L immunoadsorbent. The elution profile is shown in Figure 4. As preliminary experiments demonstrated that a significant amount of residual C_L (15%) remained unbound, the fall-through fraction was reapplied to the column a second time. The results of sequence analysis of the fraction of acid-cleaved light chain that did not bind to the immunoadsorbent after a second passage are shown in Table II. The pattern of Pth amino acids obtained at each cycle resembles that of the unmodified, uncleaved light chains. Pth amino acids arising from the constant region were not identified in this fraction. In this experiment the lower limit of detection for the stable aliphatic Pth amino acids was 0.1 nmol.

However, sequence analysis of the fraction which *bound* to the immunoadsorbent (Table II) indicated that, in addition to the constant region sequence, Pth amino acids arising from V_L were also present. Thus, the unbound fraction consisted of pure V_L while the bound fraction contained a mixture of V_L and C_L.

Isolation and Characterization of V_L from the Homogeneous Antibody 3T74. As the purification of V_L by affinity chromatography from pooled nonimmune light chains was

Table III: Sequenator Analyses of Antibody 3T74 Light-Chain Fragments[a]

fraction	Pth amino acids recovered at each cycle[b]						
	1	2	3	4	5	6	7
not bound to anti-C_L peptide immunoadsorbent	Ala (9.5)	Val (7.4)	Leu (7.0) Ala (0.7)	Thr (4.5)	Gln	Thr (2.2) Val (0.3)	Pro (1.0) Leu (0.2)
bound to anti-C_L peptide immunoadsorbent	Pro (1.3) Ala (2.6)	Val (6.8)	Ala (3.6) Leu (2.5)	Pro (1.9) Thr	Thr (1.8)	Val (3.0)	Leu (2.6)
known sequence[c] for C_L (110–)	Pro	Val	Ala	Pro	Thr	Val	Leu
known V_L sequence of 3T74 light chain[d]	Ala	Val	Leu	Thr	Gln	Thr	Pro

[a] Following cleavage at the aspartyl-109–prolyl-110 peptide bond under mild acid conditions, 3T74 light chain was fully reduced and cysteine residues were reacted with *p*-chloromercuribenzoate. The mixture was applied to a Sepharose immunoadsorbent containing sheep antibodies rendered specific for a rabbit κ light-chain constant-region peptide (residues 110–211). [b] Yields (nmol) of Pth amino acids as determined by gas–liquid chromatography are given in parentheses. [c] N-terminal amino acid sequence of the rabbit κ b4 allotype light chain beginning at position 110 was previously reported (Strosberg et al., 1972; Fraser et al., 1972). [d] Amino-terminal sequence of the antitype III pneumococcal antibody 3T74 light chain has been previously reported (Haber et al., 1977).

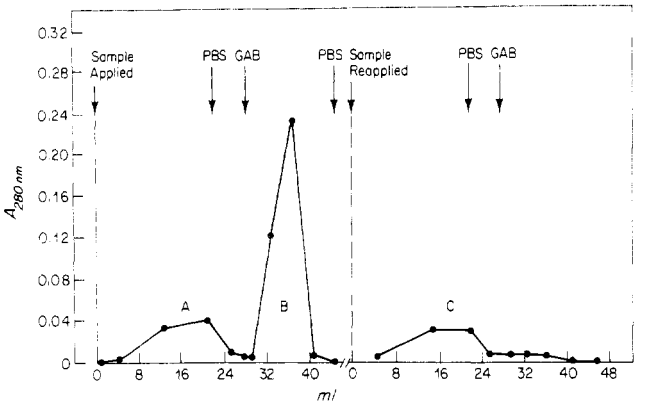

FIGURE 4: Isolation of V_L by affinity chromatography on the sheep antirabbit C_L peptide column. Acid-cleaved, fully reduced, and PCMB-treated heterogeneous b4 light chains were separated from uncleaved light chains by gel filtration on Sephadex G-75 and applied in PBS to a 4-mL column of sheep antirabbit C_L antibodies coupled to Sepharose 4B. The column was then washed with PBS, and the bound fraction (B) was eluted with GAB. The unbound fraction (A) was reapplied to the immunoadsorbent, and the washes were repeated. Fraction C contained pure V_L, while fraction B contained a mixture of V_L and C_L (see Table II).

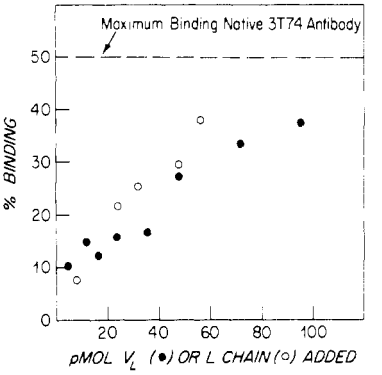

FIGURE 5: Double-antibody radioimmunoassay for binding to type III pneumococcal polysaccharide. Increasing amounts of V_L or whole light chains of the homogeneous antibody 3T74 were recombined with 32 pmol of 3T74 heavy chain in 0.01 M sodium acetate and 0.02% NaN_3, pH 5.5, for 30 min at 20 °C. The recombinants were incubated with [^{125}I]-S3-soybean trypsin inhibitor for 20 min at 20 °C. Following addition of 50 μg of normal rabbit Ig, goat antirabbit Ig was added and the precipitate and supernatant were counted. In this assay the maximum binding for native 3T74 antibody was 50%. Intact heavy chain (32 pmol) or V_L (95 pmol) alone bound less than 10%.

proven feasible, the method was then applied to the homogeneous antitype III pneumococcal polysaccharide antibody 3T74. In this experiment a preliminary separation of cleaved and uncleaved light chains by gel filtration was not done. Thus, it was expected that the fraction bound to the immunoadsorbent would contain not only C_L but also uncleaved light chains since the immunoadsorbent is expected to be specific for the sequence between residues 110 and 211. In Figure 3B $NaDodSO_4$–polyacrylamide gel patterns of the unbound and bound fractions are shown. In the unbound fraction there was a major band at ~12000 daltons. A band corresponding to a molecular size of uncleaved light chain was not found in this fraction, suggesting that all the uncleaved light chain remained bound to the immunoadsorbent. The gel pattern of the fraction remaining bound to the column showed, indeed, major bands at molecular weights corresponding to 12000 for the V_L and/or C_L and to 25000 for the intact uncleaved light chain. In both gels from bound and unbound fractions, a minor band was found with a molecular weight of 18000. This suggests that there was a minor degree of acid cleavage at a position other than that in the switch region between residues 109 and 110.

In Table III the results of amino acid sequence analysis of both the bound and unbound fractions from the immunoadsorbent using cleaved 3T74 light chain are summarized. The amino acid sequence of the unbound fraction was identical with the known 3T74 amino-terminal light-chain sequence (Haber et al., 1977). On the basis of the amounts of Pth-alanine, -valine, and -leucine detected at cycles 3, 6, and 7, respectively, the unbound fraction contained 90% V_L. The amino acid sequence of the bound fraction on the other hand contains a mixture of V_L and C_L. This was not unexpected, as in this experiment, in contrast to that using nonimmune pooled light chains, the cleaved and uncleaved light chains were not separated prior to affinity chromatography. Uncleaved light chains as well as C_L thus bind to the immunoadsorbent.

Recombination of V_L and Heavy Chain to Produce an Active Antibody Fragment. The PCMB-protected V_L from antibody 3T74 which had been isolated by using the sheep antirabbit C_L peptide immunoadsorbent was treated with dithiothreitol and the intradomain disulfide bridge re-formed. The reoxidized V_L was then recombined with heavy chain and assayed for binding capacity to type III pneumococcal polysaccharide. In Figure 5 a comparison of the antigen-binding activity of the V_L–heavy-chain recombinant and the recombinant between heavy chain and unmodified 3T74 light chain is shown. The 3T74 V_L is indistinguishable in its activity from the recombinant including the whole light chain, in that equivalent amounts of V_L and light chain increase the antigen binding by the same amount. In this experiment the degree of binding due to the V_L peptide alone or the heavy chain alone was <10%. The recovery of V_L in the course of reoxidation and reconstitution was 29%.

Discussion

A general method for isolating the V_L domain from rabbit immunoglobulins of the prevalent b4 allotype is detailed. The results demonstrate that, first, immunoglobulin domains differ enough from one another to be separated even in a denatured state by affinity chromatography using antibodies directed against one of the domains. This differs from prior methods which depended on selective enzymatic degradation of domains that were not desired. Second, it is of particular interest that the antigen-binding capacity of the V_L domain when recombined with heavy chain is equivalent to that of intact light chain. This observation supports and extends previous studies, suggesting that the constant domains are unnecessary for antigen binding. Third, an immunoglobulin domain appears to have all the necessary information within its primary sequence to refold to the correct tertiary structure from the completely denatured state. This had been shown by Hochman et al. (1976) by reoxidation of a domain in the presence of urea.

Enzymatic cleavage of human myeloma light chains in the switch region between V_L and C_L has permitted separation of the two domains by starch-block electrophoresis (Solomon & McLaughlin, 1969; Karlsson et al., 1969). However, cleavage of the rabbit κ light chain in the switch region by dilute acid treatment did not result in half-molecules until the cleaved chains were fully reduced and carboxymethylated (Poulsen et al., 1972). Amino acid sequence studies demonstrated that rabbit κ light chains contain, in addition to the two intrachain disulfide bonds in positions homologous to κ chains of other species, an extra disulfide bond spanning the variable and constant regions (Strosberg et al., 1972, 1975; Margolies et al., 1975). Thus, in order to separate V_L from C_L in rabbit κ chains, the interdomain disulfide bridge must be reduced. Preliminary experiments indicated that the interdomain disulfide bridge could not be selectively reduced, nor were the fully reduced and alkylated V_L and C_L separable by electrophoretic or ion-exchange methods (M. N. Margolies, unpublished experiments). The approach that proved successful in the present work depended on production of antibodies specific for the reduced and alkylated C_L, with subsequent purification of V_L by affinity chromatography.

Sheep antibodies raised against a mixture of fully reduced and alkylated V_L and C_L were rendered specific for C_L by purification on an immunoadsorbent containing a large C_L peptide including residues 110–211. This C_L peptide was prepared from nonimmune allotype b4 light chains by a strategy suggested by structural features of the allotype b4 κ chain (Margolies et al., 1974) outlined in Figure 1. In addition to the intradomain disulfide bridges (residues 23–88 and 134–194), rabbit κ light chains contain an extra disulfide bridge joining position 80 in the variable region with position 171 in the constant region (Strosberg et al., 1975). Cleavage under mild acidic conditions occurs in high yield between aspartic acid at position 109 and proline at position 110 in the interdomain or "switch" region (Fraser et al., 1972; Poulsen et al., 1972). Amino acid sequence studies on homogeneous rabbit antibody b4 light chains reveal only a single arginine in the constant region at position 211 and an invariant arginine in the variable region at position 61 (Chen et al., 1974; Margolies et al., 1975). Thus, tryptic digestion of the citraconylated, fully reduced, and alkylated light chain results in the production of a large peptide including residues 62–211 which may be separated from smaller arginine peptides simply by gel filtration (Freedlender & Haber, 1972; Margolies et al., 1975; Cannon et al., 1978). The high yield (65%) and purity of this peptide obtained from pooled nonimmune light chains suggest that there are few chains containing arginine in the third hypervariable region (residues 89–97).

The peptide including residues 62–211 was cleaved in acid, yielding two daughter peptides encompassing residues 62–109 and 110–211, respectively (Figure 2), as reported previously (Margolies et al., 1974). The latter peptide includes all of the constant region except for the carboxy-terminal tripeptide (212–214). Two assumptions were made in the use of this fully reduced and alkylated peptide to fractionate sheep antibodies elicited by fully reduced and alkylated cleaved light chains: (a) the 110–211 C_L peptide should have a sufficient number of antigenic sites to permit cross-reaction with a complete C_L (110–214); (b) sheep antibodies binding to this fully reduced and carboxymethylated peptide would cross-react with C_L produced from reduced and PCMB-treated light chains. These assumptions were proven correct by the observation that C_L from both reduced and PCMB-treated nonimmune pooled light chains and from homogeneous antibodies bound to the immunoadsorbent containing sheep anti-C_L peptide antibodies.

Although the present method was useful for purifying V_L, some fraction of V_L remained bound to the anti-C_L immunoadsorbent column along with C_L as shown by amino acid sequence analysis of the pooled nonimmune cleaved light chain fractions (Table II). Since the C_L peptide preparation contained 6% of a peptide beginning in the variable region at position 62, it is likely that the sheep antibodies purified utilizing the C_L peptide immunoadsorbent contain a fractional population of antibodies directed against determinants in pooled nonimmune light-chain *variable* regions. This would account for binding of some V_L. In the experiment utilizing the homogeneous antibody light-chain 3T74, however, some of the V region sequence detected in the bound fraction may be accounted for by uncleaved light chain, which was not separated from the cleaved chains before application to the immunoadsorbent column.

Two disadvantages of the reported method should be noted. First, the amount of V_L which may be prepared is limited by the low solubility of the fully reduced and PCMB-treated or fully reduced and carboxymethylated light chains. Second, as cleavage in the switch region by dilute acid is sequence specific (Asp-Pro), the method is limited to light chains of allotype b4 and b9 (Farnsworth et al., 1976); light chains of b5 and b6 allotype are not cleaved under mild acidic conditions (M. Margolies and L. E. Cannon, unpublished experiments).

By employing a reversible cysteine-protecting reagent, PCMB (Anfinsen & Haber, 1961), it was possible to reoxidize the V_L domain from the homogeneous antibody 3T74, following purification on the anti-C_L immunoadsorbent. The renatured V_L, upon recombination with the homologous heavy chain, recovered antigen-binding activity indistinguishable from that of the recombinant including intact light and heavy chains (Figure 5). A previous study showed that a V_H fragment from a homogeneous rabbit antibody was partially active in an antigen-binding assay. The V_L characterized in this work is as active as antibody light chain. In addition, the complete V_L region has been isolated since the amino terminus was detected on sequence analysis. The fraction bound to the anti-C_L immunoadsorbent contained a sequence beginning at position 110, indicating that the acid cleavage proceeded as expected, and the molecular weight was consistent with a complete domain.

The domain theory of immunoglobulins has been further extended by the present work. The V_L domain has been shown to recombine with heavy chain of an elicited homogeneous

antibody in a conformation capable of binding to a large antigen. Previous studies (Hochman et al., 1973; Sharon & Givol, 1976) utilized myeloma proteins which were found to bind a small ligand, DNP.

We previously postulated that the interdomain disulfide bridge in rabbit κ chains does not modify the folding of the variable and constant domains (Strosberg et al., 1975). This was based on the recognition that the homologous residues in the human myeloma λ-chain dimer Mcg (Schiffer et al., 1973) and the human myeloma Fab λ New (Poljak et al., 1973) approach each other by a distance compatible with the spatial requirements for disulfide bond formation. The regaining of antigen-binding activity by the V_L–heavy chain recombinant establishes that the interdomain disulfide bridge is not necessary to stabilize the V_L tertiary structure. If genes for the variable and constant regions evolved separately, it is unclear how the respective half-cysteine residues are conserved.

In conclusion, V_L from a rabbit homogeneous antibody can substitute for light chain in an antigen-binding assay and regains its native conformation from the completely denatured state. These are two of the criteria which must be fulfilled for the successful total chemical synthesis of an active immunoglobulin V_L domain. The chemical strategy and problems of such a synthesis have been discussed (Burton et al., 1977; Erickson & Krieger, 1977; Gavish et al., 1978).

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References

Anfinsen, C. B., & Haber, E. (1961) *J. Biol. Chem. 236*, 1361.
Brauer, A., Margolies, M. N., & Haber, E. (1975) *Biochemistry 14*, 3029.
Burton, J., Rosemblatt, M. S., & Haber, E. (1977) *Antibodies in Human Diagnosis and Therapy* (Haber, E., & Krause, R. M., Eds.) p 205, Raven Press, New York.
Cannon, L. E., Margolies, M. N., Strosberg, A. D., Chen, F. W., Newell, J., & Haber, E. (1976) *J. Immunol. 117*, 160.
Cannon, L. E., Margolies, M. N., & Haber, E. (1978) *Biochemistry 17*, 3101.
Chen, F. W., Cannon, L. E., Margolies, M. N., Strosberg, A. D., & Haber, E. (1976) *J. Immunol. 117*, 807.
Chen, K. C. S., Kindt, T. J., & Krause, R. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1995.
Cheng, W. C., Fraser, K. J., & Haber, E. (1973) *J. Immunol. 111*, 1677.
Edelman, G. M. (1970) *Biochemistry 9*, 3197.
Ehrlich, P. H., Matsueda, G. R., Margolies, M. N., & Haber, E. (1978) *Immunochemistry 15*, 937.
Erickson, B. W., & Krieger, D. E. (1977) *Antibodies in Human Diagnosis and Therapy* (Haber, E., & Krause, R. M., Eds.) p 159, Raven Press, New York.
Farnsworth, V., Goodfliesh, R., Rodkey, S., & Hood, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1293.
Fraser, K. J., Pulsen, K., & Haber, E. (1972) *Biochemistry 11*, 4974.
Freedlender, E., & Haber, E. (1972) *Biochemistry 11*, 2362.
Gavish, M., Zakut, R., Wilchek, M., & Givol, D. (1978) *Biochemistry 17*, 1345.
Gottlieb, A. B., Seide, R. K., & Kindt, T. J. (1975) *J. Immunol. 114*, 51.
Haber, E., Margolies, M. N., & Cannon, L. E. (1977) *Antibodies in Human Diagnosis and Therapy* (Haber, E., & Krause, R. M., Eds.) p 45, Raven Press, New York.
Hochman, J., Inbar, D., & Givol, D. (1973) *Biochemistry 12*, 1130.
Hochman, J., Gavish, M., Inbar, D., & Givol, D. (1976) *Biochemistry 15*, 2706.
Hood, L., Eichmann, K., Lackland, H., Krause, R. M., & Ohms, J. J. (1970) *Nature (London) 228*, 1040.
Jaton, J. C., Waterfield, M. D., Margolies, M. N., Bloch, K. J., & Haber, E. (1971) *Biochemistry 10*, 1583.
Kabat, E. A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1345.
Karlsson, F. A. (1971) *J. Immunol. 109*, 110.
Karlsson, F. A., Peterson, P. A., & Berggaard, I. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1257.
Lin, L.-C., & Putnam, F. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2649.
Margolies, M. N., & Brauer, A. W. (1978) *J. Chromatog. 148*, 429.
Margolies, M. N., Strosberg, A. D., Fraser, K. J., Perry, D. J., Brauer, A., & Haber, E. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 809.
Margolies, M. N., Cannon, L. E., Strosberg, A. D., & Haber, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2180.
Michaelson, T. E., Forre, O., & Natvig, J. B. (1977) *J. Immunol. 116*, 2275.
Mole, L. E., Geier, M. D., & Koshland, M. E. (1975) *J. Immunol. 114*, 1442.
Niall, H. D., & Potts, J. T. (1970) *Peptides: Chemistry and Biochemistry* (Weinstein, B., Ed.) p 215, Marcel Dekker, New York.
Poljak, R. J., Amzel, L. M., Avery, H. P., Chen, B. L., Phizackerley, R. P., & Saul, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3305.
Poulsen, K., Fraser, K. J., & Haber, E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2495.
Rodwell, J. D., & Karush, F. (1978) *J. Immunol. 121*, 1528.
Rosemblatt, M. S., & Haber, E. (1978) *Biochemistry 17*, 3877.
Schiffer, M., Girling, R. L., Ely, K. R., & Edmundson, A. B. (1973) *Biochemistry 12*, 4620.
Segal, D. M., Padlan, S. A., Cohen, G. H., Silverton., E. W., Davies, D. R., Rudikoff, S., & Potter, M. (1974) *Prog. Immunol., Proc. Int. Congr. Immunol., 2nd, 1974 1*, 93.
Sharon, J., & Givol, D. (1976) *Biochemistry 15*, 1591.
Smith, B. R., & Dorrington, K. J. (1972) *Biochem. Biophys. Res. Commun. 46*, 1061.
Solomon, A., & McLaughlin, C. (1969) *J. Biol. Chem. 244*, 3393.
Strosberg, A. D., Fraser, K., Margolies, M. N., & Haber, E. (1972) *Biochemistry 11*, 4978.
Strosberg, A. D., Margolies, M. N., & Haber, E. (1975) *J. Immunol. 115*, 1422.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53*, 624.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462.
Winkelhake, J. L. (1978) *Immunochemistry 15*, 695.