Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2569–2573, October 1971

Amino-Acid Sequences of Light Chains of a Rabbit Anti-*p*-Azobenzoate Antibody

(half-cystine peptic peptides/rabbit/N-terminal residues/hapten/bovine gammaglobulin)

E. APPELLA*, A. CHERSI*, O. A. ROHOLT†, AND D. PRESSMAN†

* National Institutes of Health, Bethesda, Maryland 20014; † Department of Biochemistry Research, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, N.Y. 14203

Communicated by Linus Pauling, July 28, 1971

ABSTRACT **Half-cystine peptic peptides representing the intrachain disulfide bonds of the light chain from an apparently homogeneous rabbit anti-*p*-azobenzoate antibody were isolated in good yields after reduction and alkylation with [^{14}C]iodoacetate, and their sequences were determined. The sequence of the first 21 amino-terminal residues was also determined. The good yields of these peptides, the fact that no variants of any of them were found, and the cleanness of the amino-terminal sequence determination confirm the high degree of homogeneity of this light-chain preparation. Previous evidence for the homogeneity of the light chain includes the appearance of only a single band upon analysis by disc electrophoresis, a relatively unique amino-acid composition, and a simple tryptic peptide map. The whole antibody shows a homogeneity of the hapten-binding constant. The antigen used in the present work is complex, since the attached hapten groups are in a large variety of environments, particularly since the carrier is a heterogeneous mixture of globulins. The very limited heterogeneity of the antibodies found in this case would appear to depend on the stimulation of only a few of the cells capable of producing antibody against a given hapten, rather than on a structural identity of the environment around each individual hapten group that is located on the antigen molecule.**

Many studies on the structures of human and mouse myeloma immunoglobulins have been reported in recent years (1–3). These proteins possess a striking uniformity and show structural features common to the immunoglobulin molecules of several species, and their study has permitted the delineation of variable and constant regions of the immunoglobulins. These variable regions are primarily responsible for differences among individual myeloma proteins and for the different specificities among individual antibodies.

The heterogeneity of most antibody preparations is well known, and because of this heterogeneity, amino-acid sequence determinations have not been possible. There are, however, reports of antibodies that appear to have restricted heterogeneity. Antihapten antibodies formed by certain individual rabbits against *p*-azobenzoate that is coupled to bovine gammaglobulin have been reported to be of very restricted heterogeneity (4, 5). Hyperimmunization of rabbits with streptococcal or pneumococcal vaccines has also resulted in a response of limited heterogeneity (6, 7).

In this report, we present the sequences of the peptic peptides that contain half-cystines and the N-terminal amino-acid sequence of the light chain of a homogeneous antibenzoate antibody prepared in the rabbit. A major factor in the success of the determination was the homogeneity of the light chain.

MATERIALS AND METHODS

Anti-*p*-azobenzoate antibodies were isolated from the immune serum of an individual rabbit (no. 2717) after bleedings spaced over several months, interspersed with intravenous injections of diazotized *p*-aminobenzoic acid conjugated with bovine gammaglobulin (4). The antibody was specifically purified by means of a solid adsorbent, consisting of diazotized *p*-aminobenzoic acid coupled to a polymer of rabbit-serum albumin. The antibody was homozygous for the a1b4 allotypes. Normal IgG from serum pools of uninjected b4 homozygous rabbits was prepared as was described (8). The light and heavy chains of the antibody to *p*-azobenzoate were separated by gel filtration on Sephadex G-100 in 1 M propionic acid after reduction of the antibody with 0.02 M dithiothreitol and alkylation with an equivalent of iodoacetamide (4). The chains from normal IgG were separated by gel filtration on Sephadex G-100 in 6 M urea containing 0.05 M formic acid, after oxidative sulfitolysis of interchain disulfide bonds (8). The N-terminal amino-acid sequences of the light chains were determined in a Beckman model 890 protein sequencer, and the resulting phenylthiohydantoin derivatives were identified by gas chromatography (9). Half-cystine residues were labeled with [^{14}C]iodoacetic acid after reduction in 5 M guanidine, and 0.05 M Tris (pH 8.2). Fully carboxymethylated light chains were digested with pepsin at an enzyme to substrate ratio of 1:50 in 5% formic acid for 12 hr, after which the digest was frozen and lyophilized. For isolation of the peptides that contain half-cystines, the peptic digest was dissolved in 0.05 M ammonia and passed through a G-25 Sephadex column (0.9 × 170 cm). The labeled fractions obtained were pooled and dried. In the case of the normal light chains, the pools were further fractionated on a Dowex 50-X2 (200–400 mesh) column (1 × 20 cm), and developed with a linear gradient of 0.2–2.0 M pyridine–acetic acid buffer (pH 3.1–5.0). The radioactive fractions were further purified by high-voltage paper electrophoresis at pH 6.5 and at pH 3.6. For light chains from rabbit 2717, the fractionation on the column was omitted and the peptides that contain half-cystine were purified directly by high voltage paper electrophoresis. Amino-acids were analyzed with a Beckman-Spinco model 644 amino-acid analyzer. Samples were hydrolyzed under reduced pressure in 6 N HCl for 24 hr. Digestion with carboxypeptidases A and B was done as described elsewhere (10). Amino-terminal residues of peptic peptides were identified by the dansyl method of Gray and Hartley

TABLE 1. *Amino-terminal sequence of the light chain of antibody from rabbit no. 2717 and corresponding residues from mouse and human light chains*

	Residue no. 1	2	3	4	5	6	7	8	9	10	11
Rabbit 2717 light chain	Val	Glu	Val	Leu	Thr	Glx	Thr	Pro	Ser	Pro	Val
Mouse* light chains	Asp 33 Glu 5	Ile 28 Val 7 Thr 2 Asn 1	Val 22 Gln 12 Thr 2 Leu 2	Met 25 Leu 8 Val 3 Ile 2	Thr 35 Ile 3	Gln 38	Ser 29 Thr 7 Asx 2	Pro 30 Gln 3 Ser 3 Thr 1	Ala 14 Ser 12 Leu 8 Thr 2 Asn 1	Ser 25 Thr 4 Phe 4 Ile 2 Glx 2 Tyr 1	Leu 30 Met 6 Asp 2
Human† light chains	Asp 25 Glu 15	Ile 41 Val 1 Met 1	Val 19 Glu 22 Leu 1 Ile 1	Met 24 Leu 18 Val 1	Thr 40	Gln 40	Ser 38 Thr 1	Pro 40	Ser 21 Gly 10 Ala 4 Leu 3 Thr 1 Asn 1	Ser 22 Thr 17 Phe 1	Leu 40

	Residue no. 12	13	14	15	16	17	18	19	20	21
Rabbit 2717 light chain	Ser	Ala	Ala	Val	Gly	Gly	Thr	Val	Thr	Ile
Mouse* light chains	Ser 24 Ala 7 Pro 4 Phe 1 Gln 1	Val 22 Ala 12 Met 2 Thr 2	Ser 28 Thr 8 Ala 2	Leu 14 Val 7 Ile 6 Ala 5 Pro 3 Ser 2 Met 1	Gly 36 Ser 2	Glu 18 Asp 10 Gln 4 Gly 3 Lys 2	Arg 13 Lys 8 Glx 6 Thr 6 Ser 2 Pro 2	Val 29 Ala 9	Thr 23 Ser 13	Ile 25 Leu 5 Met 6 Val 1
Human† light chains	Ser 34 Pro 4	Ala 19 Leu 12 Val 6	Ser 34 Thr 4	Val 20 Pro 17 Leu 1	Gly 37 Arg 1	Asp 22 Glu 16 Gln 1	Arg 34 Pro 4	Val 17 Ala 14 Ile 1	Thr 25 Ser 4 Ala 3	Ile 18 Leu 10 Val 1

* Data from Hood *et al.* (15) and Appella (unpublished). Each number represents the occurrence of the indicated residue for 38 mouse light-chain preparations.

† Data from Wu and Kabat (14). Each number represents the occurrence of the indicated residue for 40 human light-chain preparations.

(11), and the amino-acid sequences were determined by the dansyl or subtractive Edman procedures (12).

RESULTS

The sequence of the first 21 residues at the amino-terminal portion of the light chains of anti-*p*-azobenzoate antibody from rabbit no. 2717 are shown in Table 1. At each position a single major amino acid was found in a yield of 40–80%. This is in contrast to the data obtained from light chains derived from nonimmune gammaglobulins of the same allotypic specificity (b4), where several major amino-acid alternatives appear in each position (13).

The sequence of the N-terminal portion of the light chains of antibody no. 2717 is quite different from that of any of the 40 human or 38 mouse myeloma light chains so far described (14, 15,‡) (Table 1). Indeed, valine was found in position 1 for light-chain 2717, and has not been reported previously for any myeloma light chain. Similarly, glutamic acid in position 2, proline in 10, and valine in 11 were unique to the antibody-light chain 2717. Alanine was found in position 14 in only two of the 78 light chains and glycine in position 17 in only three light chains.

The peptides that contain half-cystine were also isolated from light chains 2717 and characterized. The light chains were carboxymethylated with [^{14}C]iodoacetic acid and digested with pepsin. The digest was fractionated on a Sephadex G-25 column, and two sharp, reasonably well-separated peaks of radioactivity appeared. The fractions were combined accordingly to give pools 1 and 2, in the order of elution. These pools were subjected to paper electrophoresis at pH 6.5. The patterns of an analytical run are shown in Fig. 1.

The peptides separated well as indicated by ninhydrin staining. All the bands were assayed for ^{14}C in a liquid scintillation counter. The regions between the bands were also assayed. The radioactive peptides are indicated in Fig. 1. All the peptides except 1-3a and 1-3b were further purified by paper electrophoresis at pH 3.6 or at pH 1.9.

‡ Appella, E., submitted for publication.

TABLE 2. *Amino-acid composition of peptic peptides from [^{14}C]carboxymethylated light chains of rabbit 2717*

	Peptide							
	1-1	1-2	1-3a	1-3b	2-1	2-2	2-3a	2-3b
Lys	2.0	1.0						
His	0.9							
CMC	0.7	0.7	0.6	0.8	0.7	0.7	0.6	0.6
Asp	0.9	3.1	1.9	1.9	0.8	1.0		
Thr	3.6	1.0	1.0			1.0		
Ser	2.2	2.7						1.0
Glu	2.1	0.9	1.0	1.0				
Gly	1.1				2.0			
Ala			2.0		1.0			
Val	0.8						1.0	
Ile		1.4					1.0	1.0
Leu						1.0		
Tyr	1.5	1.0			2.6	0.8		
Yields (%)	27	52	26	12	13	24	27	24
Mobility*	+0.24	−0.08	−1.05	−1.20	−0.20	−0.32	−0.44	−0.44
N-terminal	Thr	Ser	Glu	Glu	Tyr	Asp	Ile	Ile

* The positive mobilities are expressed relative to lysine, and the negative mobilities relative to aspartic acid.

The yields of the radioactive peptides and their amino-acid compositions are given in Table 2.

Eight radioactive peptides were isolated in good yield. Two of them, 1–3b and 2-2, were shown by sequence analysis to be smaller fragments of larger peptides, 1-3a and 1-2, respectively. Thus, the eight peptides represent six half-cystine-containing sequences. The seventh half-cystine, present on the basis of the amino-acid analysis of the light chain, is the carboxyl-terminal one, which had been alkylated by the unlabeled iodoacetic acid preparatory to the chain separation, and thus did not appear as a radioactive band.

In the case of normal light chains of b4 allotypic specificity, the majority have also been shown to contain seven half-cystines by amino-acid analysis (8). However, the isolation of labeled peptides from normal light chains after full carboxymethylation with [^{14}C]iodoacetic acid and peptic digestion yielded many more peptides than six, indicating the heterogeneity of these light-chain preparations. Nevertheless, peptides corresponding to the peptides 1-1, 2-2, and 2-3a of the light chains of the antibody (Table 2) were obtained from the normal light chains in good yield, signifying the conservative nature of the corresponding regions of the peptide chains. Other peptides that contain half-cystine were obtained in low yields from the normal light chains, indicating the heterogeneity of the normal light chains. The sequences of several of these peptides were determined, and some of these could be identified as homologs of peptides isolated from the 2717 preparation. The sequences of these peptides are shown in Table 3.

Peptides 2-3a and 1-1 have been isolated in high yield from b4 light chains of several different normal light-chain pools. They are identical to the peptides from 2717-b4 chains, and thus appear to represent peptides of the constant region. Peptide 2-3a is not related to peptide 2-3b although they differ only by the second of three residues. Peptide 2-3a can be isolated from one large tryptic peptide, which does not yield peptide 2-3b, while only peptide 2-3b can be isolated from a different large tryptic peptide (Appella *et al.*, unpublished data).

Peptide 2-3b appears to correspond to positions 21, 22, and 23 of light-chain 2717 sequence on the basis that it was the only radioactive half-cystine-containing peptide which was isolated from a peptic digest of the amino-terminal tryptic peptide (Appella *et al.*, unpublished data).

Peptide 2-2 and peptide 1-2 appear to result from differences in the peptic splitting of a sequence that is common to both light-chain preparations. Peptide 1-2 is of particular interest. Frangione and Lamm (16) reported that for this sequence, alanine in b4 light chains is replaced by aspartic acid in b5 light chains, and they assigned this difference as a structural basis for b4–b5 allotypy. Contrary to this assignment, we find that peptide 1-2 from light-chain 2717 contains no alanine, while the homologous peptide 1-2 from the normal light chains contains alanine, and both preparations are of homozygous b4 allotype.

Peptide 2-1 contains the very characteristic Tyr–Tyr–Cys sequence, and from homology with sequences of mouse and human kappa chains, peptide 2-1 must include positions 86–88, generally assigned to the Tyr–Tyr–Cys sequence. This peptide is from a region that is hypervariable in rabbit (16), as well as in man and mouse. Although peptide 2-1 is from a hypervariable region, it is homogeneous, further emphasizing the homogeneity of light chain 2717.

The position of peptide 1-3 cannot yet be fixed and does not appear to carry any differences related to allotypes.

DISCUSSION

The light chain of antibody 2717 that was studied here has been found to be homogeneous. Evidence for homogeneity is the following: (*a*) At each position in the amino-terminal portion of the molecule, a single major amino acid was obtained in good yield. (*b*) Six internal half-cystine sequences were identified. The identifying peptides were obtained in

TABLE 3. *Comparison of sequences of peptic peptides from carboxymethylated rabbit light chains*

	2717	Thr(Ser Thr Glx Tyr Asx Ser His)Lys Gln Tyr Thr Cys Lys(Gly Thr)Val
Peptide 1-1		
	b4b4	Thr Ser Thr Glu Tyr Asx Ser His Lys Gln Tyr Thr Cys Lys(Gly Thr)Val
	2717	Ser(Lys Ile Ser Glx Asx Ser Asx)Asp Cys Thr Tyr
Peptide 1-2		
	b4b4	Ser Lys Thr Pro Glu Asp Ser Ala Asp Cys Thr Tyr
	2717	Glu Cys Asx Asx Ala Ala Thr
Peptide 1-3		
	b4b4 variant	Glu Cys Ala/Asp Asp Ala Ala Thr
	2717	Tyr Tyr Cys(Gly Gly Ala)Asx Tyr
Peptide 2-1		Tyr Tyr Cys Gln Gln Gly Ser Tyr
	b4b4 variants*	Tyr Tyr Cys Gln Gly Ser/Ala
		Tyr Tyr Cys Gln Gln Ser Ala/Gly
	2717	Asp Cys Thr Tyr Leu
Peptide 2-2		
	b4b4	Asp Cys Thr Tyr Leu
	2717	Ile Val Cys
Peptide 2-3a		
	b4b4	Ile Val Cys
	2717	Ile Ser Cys
Peptide 2-3b		
	b4b4 variants	Ile Asn/Ser Cys

* These sequences are based on data obtained by Frangione and Lamm (1970).

good yield without evidence of variants. (*c*) The light chain moves as a single band upon analysis by disc electrophoresis (4). (*d*) The intact antibody shows a very simple pattern that was detected by isoelectric focusing (17). (*e*) The intact antibody shows homogeneity with respect to binding constant, which was determined at various hapten concentrations (4).

On the other hand, the light chains of pooled normal IgG of the same allotype was a mixture. The N-terminal sequence showed many variants, and there were also many peptides that contain half-cystine, an observation that also indicates variants. However, three peptides that contain half-cystine were obtained in good yield (peptides 1-1, 1-2, and 2-3a). These would appear to represent peptides of constant regions, particularly, since these same peptides were also isolated from the antibody light chain. The number of variants found for peptides 1-3 and 2-3b seem to be limited, compared to the number of variants of peptide 2-1. Since the assignment of peptide 2-1 involves half-cystine 88, it appears to come from a hypervariable region of the light chain, while the regions involving peptides 2-3b and 1-3 are relatively conservative. These observations parallel similar observations made by Porter for rabbit heavy chain where the hypervariable region seems to be located in the region around 100–110 (18).

The amino-terminal sequence does not appear to contribute to the combining site specificity. The light chain of the antibody to *p*-azobenzoate produced in rabbit 2717 shows a difference of only four residues in the first 21 amino-terminal residues, when compared with a light chain of an antibody to benzenearsonate, also of restricted heterogeneity (Appella *et al.*, unpublished data).

Moreover considerable homology seems to exist between the N-terminal sequences of kappa light chains from mouse, human, and rabbit. Aside from the initial three residues, where there is indication of some complexity due to varying chain lengths, the only residues not common to the rabbit, human, and mouse are in positions 10 and 11. It is of particular interest that for 17 residues, the N-terminal sequence of the light chain of an antibody to group-C streptococci is identical to an anti-S8 antibody, except for an additional alanine residue at the N-terminus.

In each of the light chains of human (κ and λ) and mouse (κ), subgroups have been established on the basis of the homology of the amino-terminal amino-acid sequence. The amino-terminal sequences of several rabbit kappa chains from an antibody of restricted heterogeneity are now available. If the amino-terminal alanine in the light chains of the antibody to group-C streptococci (19) is counted as an extra amino-terminal residue, then the remaining amino-terminal amino-acid sequence is homologous to that of the light chain of anti-pneumococcal antibody (20) for at least 16 residues, and both are similar to that of subgroup III of human kappa chains (19). Therefore, these light chains may represent a single subgroup.

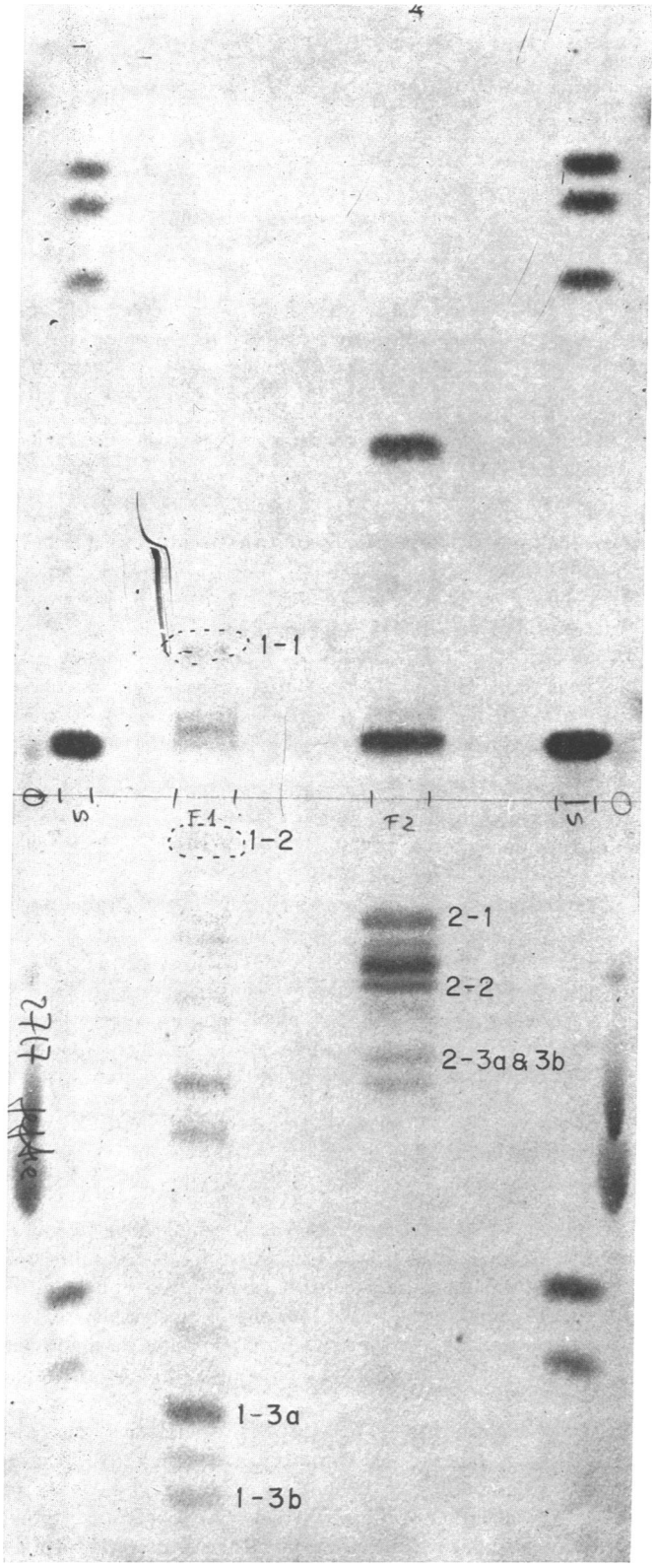

FIG. 1. Separation of peptic peptides in pool 1 (*left center*) and pool 2 (*right center*) by paper electrophoresis at pH 6.4 and 3000 V for 50 min. The patterns on the edges are for the standard amino-acid mixture and dyes used to calculate the mobilities.

However, the N-terminal sequence of light chain 2717 (Table 1) is greatly different (7 amino acids out of at least 16) from that of the light chain of the anti-group C and the anti-pneumococcal rabbit antibodies. Thus, light chain 2717 represents a different subgroup.

Although antigen heterogeneity has been emphasized as an important cause of antibody heterogeneity, it should be noted that the antigen used in the present work is particularly complex and heterogeneous, since the carrier is a heterogeneous mixture of globulins, and the attached groups must be in a large variety of environments, even on the same carrier globulin. The very limited heterogeneity of the antibodies found in this case would appear to depend on the stimulation of only a few cells capable of producing antibodies against a given hapten, rather than on a structural identity of the environment around each individual haptenic group that is located on the antigen molecule (4).

We thank Mr. Gerald Radzimski for his technical assistance and Dr. R. G. Mage for the allotyping. This work was supported in part by Grant no. AI 3962 from the National Institute for Allergy and Infectious Diseases.

1. Reviewed in *Cold Spring Harbor Symp. Quant. Biol.*, **32** (1967).
2. *Symposium on Developmental Aspects of Antibody Formation and Structure*, eds. J. Sterzl and H. Rhia (Academic Press, New York, 1969).
3. Milstein, C., and J. Pink, *Progr. Biophys. Mol. Biol.*, **21**, 211 (1970).
4. Roholt, O. A., B. K. Seon, and D. Pressman, *Immunochemistry*, **7**, 329 (1970).
5. Seon, B. K., O. A. Roholt, and D. Pressman, *Immunochemistry*, in press.
6. Braun, D. G., K. Eichmann, and R. M. Krause, *J. Exp. Med.*, **129**, 809 (1969).
7. Pincus, J. H., J. C. Jaton, K. J. Bloch, and E. Haber, *J. Immunol.*, **104**, 1149 (1970).
8. Rejnek, J., E. Appella, R. G. Mage, and R. A. Reisfeld, *Biochemistry*, **8**, 2712 (1970).
9. Pisano, J., and T. Bronzert, *J. Biol. Chem.*, **231**, 393 (1969).
10. Appella, E., R. G. Mage, S. Dubiski, and R. A. Reisfeld, *Proc. Nat. Acad. Sci. USA*, **60**, 975 (1968).
11. Gray, W. R., and B. S. Hartley, *Biochem. J.*, **89**, 379 (1963).
12. Konigsberg, W., and R. J. Hill, *J. Biol. Chem.*, **238**, 2547 (1962).
13. Jaton, J. C., M. D. Waterfield, M. N. Margolies, and E. Haber, *Proc. Nat. Acad. Sci. USA*, **66**, 959 (1970).
14. Wu, T. T., and E. A. Kabat, *J. Exp. Med.*, **132**, 211 (1970).
15. Hood, L., M. Potter, and D. J. McKean, *Science*, **170**, 1207 (1970).
16. Frangione, B., and M. E. Lamm, *FEBS Lett.*, **11**, 339 (1970).
17. Hoffman, D. R., A. L. Grossberg, and D. Pressman, *Immunochemistry*, in press.
18. Fruchter, R. G., S. A. Jackson, L. E. Mole, and R. R. Porter, *Biochem. J.*, **116**, 249 (1970).
19. Hood, L., K. Eichmann, H. Lackland, R. M. Krause, and J. J. Ohms, *Nature*, **228**, 1040 (1970).
20. Jaton, J. C., M. D. Waterfield, M. N. Margolies, K. J. Bloch, and E. Haber, *Biochemistry*, **10**, 1583 (1971).