easily derived by expressing the standard moments as ratios of radial derivatives of the concentration. Then

$$M_{z+1} = \frac{d(cM_wM_z)}{d(cM_w)} \quad (A10)$$

Combination of eq A9 and A10 then yields the desired eq 12c.

Next, we wish to obtain the derivative relations of eq 11 that relate the concentration dependence of one ideal moment to the next higher moment. From eq 12a we find that

$$\frac{d[c/M_{y1}(c)]}{dc} = \frac{2d[c/M_n(c)]}{dc} - \frac{d[c/M_w(c)]}{dc} = \frac{2}{M_w} - \frac{1}{M_v} \quad (A11)$$

where the equality on the right results from eq 10 and 12. A comparison of this result to eq A8 immediately verifies eq 11.

Finally, in order to demonstrate eq 11b, we start with eq 12b, and obtain the result

$$\frac{d[c/M_{y2}]}{dc} = \frac{M_z}{M_w^2} + \frac{1}{M_w^2 d\ln c}\frac{dM_z}{} - 2\frac{M_z}{M_w^3 d\ln c}\frac{dM_w}{} \quad (A12)$$

Substitution of eq A6 and A10 then gives us

$$\frac{d[c/M_{y2}]}{dc} = \frac{M_z}{M_w^3}(M_{z+1} - 3M_Z + 3M_w) \quad (A13)$$

and a comparison of eq A13 and 12c leads to the desired relation, eq 11b.

References

Adams, E. T., Jr. (1965a), *Biochemistry 4*, 1655.
Adams, E. T., Jr. (1965b), *Biochemistry 4*, 1646.
Adams, E. T., Jr. (1967), *Biochemistry 6*, 1864.
Adams, E. T., Jr., and Filmer, D. L. (1966), *Biochemistry 5*, 2971.
Adams, E. T., Jr., and Williams, J. W. (1964), *J. Amer. Chem. Soc. 86*, 3454.
Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.
Haschemeyer, R. H., and Bowers, W. F. (1970), *Biochemistry 9*, 435.
Johnson, M. L., and Yphantis, D. A. (1971), *Abstr. 15th Annu. Meeting Biophys. Soc.*, WPM-H18.
Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 164*, 245.
Roark, D. E., and Yphantis, D. A. (1971), *Biochemistry 10*, 3241.
Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.
Squire, P. G., and Li, C. H. (1961), *J. Amer. Chem. Soc. 83*, 3521.
Stafford, W. F., III, and Yphantis, D. A. (1972), *Biophys. J.* (in press).
Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.
Steiner, R. F. (1953a), *Arch. Biochem. Biophys. 44*, 120.
Steiner, R. F. (1953b), *Arch. Biochem. Biophys. 47*, 56.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 146*, 66.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Amino Acid Sequence of Monkey Amyloid Protein A†

Mark A. Hermodson,* Robert W. Kuhn, Kenneth A. Walsh, Hans Neurath, Nils Eriksen, and Earl P. Benditt

ABSTRACT: The amino acid sequence of amyloid protein A was determined. This protein was isolated from the liver of a monkey (*Macaca mulatta*) afflicted with amyloidosis. Amyloid protein A contains 76 amino acid residues in a single polypeptide chain devoid of disulfide bonds. Sequenator analysis of the whole protein and of a large fragment derived by cleavage with cyanogen bromide established the sequence of all but the six carboxyl-terminal residues. These were ordered by degradation with yeast protease C and by manual Edman degradation. The amino acid sequence of amyloid protein A bears no resemblance to the sequence of any protein of known function.

Amyloid substance is a complex proteinaceous material found in various tissues of patients and animals with the disease amyloidosis. Recent evidence indicates that there are two chemically distinct classes of amyloid substance: one (class A) occurs in individuals with any of several chronic inflammatory conditions while the other (class B) occurs in individuals with tumors or with no preexistent disease (Benditt and Eriksen, 1971).

A major protein constituent of the amyloid substance in inflammation-related amyloidosis has been designated "amyloid protein A." We have recently reported the amino-terminal sequence of this protein isolated from the livers of a human and a monkey afflicted with chronic inflammatory disease (Benditt *et al.*, 1971). Proteins from these two species show a high degree of sequence identity: they differ in only two positions in the 24 amino-terminal amino acid residues

† From the Departments of Biochemistry and Pathology, University of Washington School of Medicine, Seattle, Washington 98195. *Received April 13, 1972.* This work has been supported by research grants from the National Institutes of Health (GM-15731, HE-03174, and GM-13543) and the American Cancer Society (NP-18N).

and these substitutions are of conservative character (two phenylalanine residues in the human replacing a tyrosine and and a tryptophan residue in the monkey protein).

The amino acid sequence of monkey amyloid protein A is described in the present communication.

Experimental Procedure

Materials and Methods. Amyloid substance was extracted and purified from the frozen liver of a monkey (*Macaca mulatta*) afflicted with a chronic inflammatory disease resembling rheumatoid arthritis. The material and information on the monkey were kindly supplied by Drs. B. Ruebener, E. K. Smith, and R. Stowell of the National Primate Center, Davis, Calif. Amyloid protein A (50 mg) was extracted and purified from the amyloid substance as described in the previous communication (Benditt *et al.*, 1971).

Yeast protease C was prepared from Baker's yeast by the method of Kuhn *et al.* (1972). No measurable endopeptidase activity was evident when tested on the B chain of oxidized bovine insulin.

Automated Edman degradations were performed with a Beckman Sequencer Model 890A modified to allow nitrogen purging of the reaction cell during the fine vacuum steps. The mode of operation of the instrument and the methods of sequenator analysis are adaptations (by Hermodson *et al.*, 1972) of the technique of Edman and Begg (1967).

Manuel Edman degradations were performed by the following method which employed gas chromatography (Pisano and Bronzert, 1969) for the final identification of the phenylthiohydantoins. The peptide (0.15 μmole) was dissolved in 0.5 ml of buffer containing 5% (v/v) *N*-ethylmorpholine (Pierce Sequenal grade) in 50% aqueous pyridine (Pierce Sequenal grade) titrated to pH 9.6 with glacial acetic acid. Phenyl isothiocyanate (5 μl) (Baker, vacuum distilled at ~1 mm) was added, and the mixture was agitated on a Vortex mixer and then incubated under argon for 30 min at 50°. The mixture was extracted twice with 1-ml portions of benzene (Burdick and Jackson, "glass-distilled" grade) and the water layer dried thoroughly on a Buchler Evapomix. Trifluoroacetic acid (0.3 ml, Pierce Sequenal grade) was then added to the residue and incubated at 50° for 10 min. The trifluoroacetic acid was removed in a stream of argon and 0.5 ml of water was added to the residue. This mixture was extracted twice with 1-ml portions of chlorobutane (Burdick and Jackson, "glass-distilled" grade) containing 2% (v/v) ethanethiol (Eastman). The chlorobutane extracts were treated with 1 N HCl for 10 min at 80° to convert the products to the phenylthiohydantoin amino acids (Edman and Begg, 1967). The HCl solutions were extracted with ethyl acetate, and the ethyl acetate layers were analyzed by gas chromatography using the general procedure of Pisano and Bronzert (1969) as adapted by Hermodson *et al.* (1972). The water layer was dried thoroughly and coupling buffer was again added. Samples to be hydrolyzed for subtractive Edman analyses were removed at this point.

Carboxyl-terminal analyses were performed with yeast protease C (Kuhn *et al.*, 1972). Amyloid protein A (6.4 mg) was dissolved in 3 ml of 0.05 M acetic acid containing 1.5 μmoles of norleucine. The pH of the solution was adjusted to 4.15 by the dropwise addition of 4% (v/v) aqueous pyridine. Digestion at 25° was initiated by the addition of 1.51 nmoles of yeast protease C dissolved in 0.075 ml of water. Aliquots (0.2 ml) were with drawn at zero time and at appropriate time intervals and diluted with 1 ml of 10% trichloroacetic acid to stop the reaction. The precipitate was removed by centrifugation; the supernatant was extracted with ether and dried. The sample was then dissolved in 1 ml of 0.2 M sodium citrate (pH 2.2), and amino acid analysis was performed. For each time sample an enzyme blank (lacking protein) and a substrate blank (lacking enzyme) were analyzed in the same manner. Values obtained from these blanks were subtracted from those of the whole digest.

Cleavage at methionine residues was effected by treating 29 mg of protein dissolved in 1 ml of 70% aqueous formic acid with 60 mg of cyanogen bromide. The reaction was allowed to proceed at room temperature for 17 hr. The mixture was then diluted with water and lyophilized.

Results

According to amino acid analysis (Table I) amyloid protein A contains two methionine residues. Sequenator analysis of 5 mg of the whole protein (Benditt *et al.*, 1971) has placed these methionines at residues 17 and 24. Accordingly, cleavage with cyanogen bromide should yield three fragments comprising, respectively, residues 1–17, 18–24, and 25–76 (Figure 1). Since the sequence of residues 1–24 has already been reported (Benditt *et al.*, 1971), only the carboxyl-terminal cyanogen bromide fragment (residues 25–76) required analysis in order to complete the amino acid sequence of the protein.

Gel filtration of the whole cyanogen bromide digest on Sephadex G-25 yielded five peak fractions (Figure 2). According to amino acid analyses, fraction II corresponded to residues 1–17, fraction V to residues 18–24, and fraction I to the carboxyl-terminal fragment (residues 25–76). Fraction III was identical in composition with fraction II except for the lack of one (amino-terminal) arginine. Fraction IV contained small amounts of fractions III and V and no other detectable peptides. On the basis of these data, the linear alignment of these fractions in the whole protein must be II-V-I. This sequence was further proven by extending the sequenator analysis of the whole protein through the first 37 amino-terminal residues as shown in Figure 1. The sequence of residues 25–37 was identical with that of the first 13 residues of fraction I.

Sequenator analysis of 10 mg of fraction I established the positions of residues 25–70 and left only residues 71–76 to be ordered (Figure 1). The composition of these six residues was calculated by subtracting the composition of residues 25–70 from the compsition of fraction I (Table I). This procedure established the composition of the carboxyl-terminal hexapeptide as Asp, Thr, Glu, Gly, Ala, His.

Fraction I was digested at pH 8 with trypsin (Worthington) which had previously been treated with tosyl-L-phenylalanyl chloromethyl ketone. The pH was maintained in a pH-Stat at 37°. Initially the weight ratio of peptide (9 mg) to trypsin was 20:1 but a second aliquot of enzyme was added after 15 min of digestion to adjust the peptide to trypsin ratio to 10:1. After an additional 90 min, the mixture was lyophilized. Peptides were separated on a 1 × 85 cm column of Dowex 1-X-2 using a four-chamber gradient. Each chamber contained 150 ml of the following solutions: chamber 1, 0.36 M pyridine; chamber 2, 0.5 M pyridine–0.1 M acetic acid (pH 6); chamber 3, 1.0 M pyridine–0.2 M acetic acid (pH 5.5); chamber 4, 2.0 M pyridine–1.0 M acetic (pH 5.5). The flow rate was 30 ml/hr. The column effluent was continuously monitored by following the ninhydrin color of an alkaline hydrolysate of 10% of the effluent stream.

Eight tryptic peptide peaks were obtained and were num-

TABLE I: Amino Acid Composition of Amyloid Protein A and Its Cyanogen Bromide Fragments.

Amino Acid	Whole Protein[a]	Found in Sequence	CNBr Fraction I[b]	Found in Sequence	CNBr Fraction II[b]	CNBr Fraction III[b]	Found in Sequence	CNBr Fraction V[b]	Found in Sequence
Asp	10.99	11	8.41	8	2.07	1.97	2	0.99	1
Thr	0.92	1	1.03	1					
Ser	4.90	5	2.13	2	1.92	2.19	2	0.87	1
Glu	7.04	7	5.96	6	1.00	1.04	1		
Pro	1.14	1	0.89	1					
Gly	8.19	8	6.13	6	1.98	2.11	2		
Ala	11.15	11	7.81	8	2.02	2.13	2	1.01	1
Val	1.57	2	1.08	2					
Met	1.81	2							
Ile	1.58	2	1.20	2					
Leu	2.99	3	1.92	2	0.87	0.85	1		
Tyr	4.49	5	2.80	3	0.95	0.93	1	0.93	1
Phe	2.85	3	0.93	1	1.65	1.65	2		
Trp[c]	2.10	3		1			1		1
Lys	4.05	4	3.91	4					
His	1.88	2	1.90	2					
Arg	5.23	5 or 6[d]	3.20	3	1.95	1.01	2	0.93	1
Hse					0.96	1.06	1	0.76	1
Total[e]		76		52			17		7
Residue numbers		1–76		25–76			1–17		18–24

[a] Amino acid compositions are compared with the number of residues of each amino acid found in the sequence of the respective polypeptide. Monkey amyloid protein A contains no cystine or cysteine (Benditt *et al.*, 1971). Based on 24- and 48-hr hydrolyses in 3 M *p*-toluenesulfonic acid at 110° (Liu and Chang, 1971). Serine and threonine were extrapolated to zero time. Valine and isoleucine values are for the 48-hr hydrolysate. The Val_{58}–Ile_{59} bond would be expected not to hydrolyze completely in this time period. [b] Single hydrolyses for 24 hr at 110° in 6 N HCl. The Val_{58}–Ile_{59} bond would not be hydrolyzed in this time period. [c] All of the cyanogen bromide fragments gave a positive tryptophan reaction with *p*-dimethylaminobenzaldehyde. [d] Twenty-two per cent of the protein preparation lacks the amino-terminal arginyl residue (Benditt *et al.*, 1971). [e] No galactosamine or glucosamine were detected by amino acid analysis.

bered in the order of their elution from the column, T-1 to T-8. The amino acid composition of each peptide was determined (Table II). Seven peptides were obtained in 40–70% yield and an eighth peptide (T-5) in 15% yield. T-5 was identical with the amino-terminal tryptic peptide (T-1) but lacked one of the two lysyl residues. Apparently the amino-terminal lysine was cleaved from T-1 in low yield to generate T-5. All peptides were pure except T-2 which corresponded in composition to an equal mixture of peptides comprising residues 31–39 and 35–39, respectively. Apparently the bond Lys_{34}–Tyr_{35} was incompletely split by trypsin, possibly due to the negative charge of the adjacent residue, Asp_{33}.

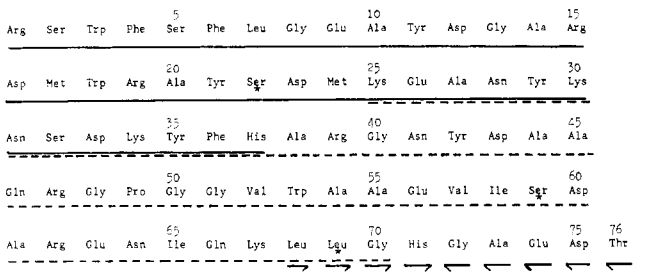

FIGURE 1: The amino acid sequence of monkey amyloid protein A. Data from the sequenator analysis of the whole protein are underlined with a solid line and those from sequenator analysis of the carboxyl-terminal cyanogen bromide fragment with a broken line. Asterisks denote residues whose identification was only tentative by sequenator analysis. Serine residues 22 and 59 were confirmed by amino acid analyses of fraction V and tryptic peptide T-7, respectively (Tables I and II). Residue 69 was confirmed as leucine by manual Edman degradations of the carboxyl-terminal tryptic peptide T-8 (indicated by →). Results of digestion of whole amyloid protein A with yeast protease C are indicated by ←

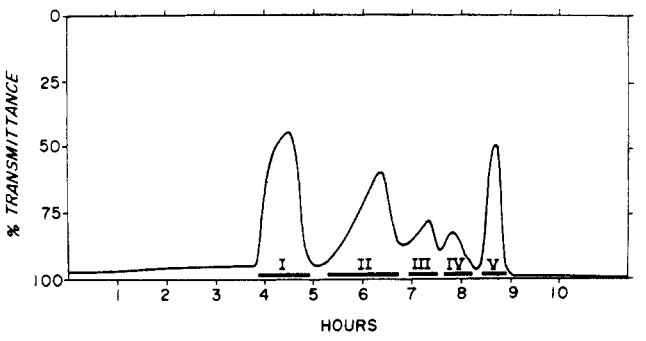

FIGURE 2: Separation of the cyanogen bromide fragments of monkey amyloid protein A on a 1 × 150 cm column of Sephadex G-25 equilibrated and developed with 10% (v/v) aqueous acetic acid at a flow rate of approximately 9 ml/hr.

TABLE II: Tryptic Peptides of Cyanogen Bromide Fraction I of Amyloid Protein A.[a]

Amino Acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7[c]	T-8
Asp	0.98	1.06	1.07	1.94	1.12	2.08	1.01	1.00
Thr								0.88
Ser		0.49			0.29	0.98	0.88	
Glu	1.00		2.07	1.08	1.00		1.03	1.06
Pro							0.75	
Gly				1.22			3.00	2.08
Ala	1.15	1.25		2.08	1.17		3.00	1.23
Val							1.37	
Ile			0.93				0.53	
Leu								1.84
Tyr	0.83	1.05		0.77	0.65			
Phe		0.68						
Lys	2.00	0.70	0.93		0.78	0.95		
His		1.00						0.98
Arg		1.05		0.91			0.77	
% yield	60	44[b]	61	40	15	43	70	62
Residue numbers	25–30	See text	63–67	40–47	26–30	31–34	48–62	68–76

[a] The data represent the results of 24-hr hydrolyses in 6 N HCl. The Val–Ile bond in T-7 was not completely hydrolyzed under these conditions. [b] Based on the quantity of histidine. [c] Only peptide T-7 gave a positive tryptophan test with *p*-dimethylaminobenzaldehyde.

The electrophoretic mobilities of the various tryptic peptides at pH 6.5 were in accord with the amide placements derived from the sequenator data, confirming the identity of the amides in the first 70 residues.

The amino acid composition of the carboxyl-terminal tryptic peptide (residues 68–76) could be predicted by subtracting the composition of residues 25–67 from the composition of the whole fraction I. This value agreed with the composition of peptide T-8 and placed this peptide at the carboxyl terminus. The peptide was strongly acidic at pH 6.5 and the amino acid analysis showed no ammonia, indicating that the the glutamyl and aspartyl residues were in the form of free acids.

Manual Edman degradations of the tryptic peptide T-8 and gas chromatographic analysis of the phenylthiohydantoins of the amino acids yielded the amino-terminal sequence Leu-Leu-Gly-, confirming the sequenator analysis. However, analysis of the fourth cycle of the degradation by gas chromatography showed no new phenylthiohydantoin. Hydrolysis of an aliquot of the peptide remaining after four cycles gave one residue each of aspartic acid, threonine, glutamic acid, and alanine, approximately 1.3 residues of glycine, and 0.3 residue of histidine. A fifth cycle of the degradation left one residue each of aspartic acid, threonine, glutamic acid, alanine, and glycine, and only a trace of histidine. Thus four residues could be placed as the sequence Leu-Leu-Gly-His.

Carboxypeptidase A digestion at pH 8.5 of the peptide T-8 released no amino acid residues. Digestion of whole amyloid protein A with yeast protease C at pH 4 proceeded smoothly to the glycine residue and yielded a definitive carboxyl-terminal sequence -Gly-Ala-Glu-Asp-Thr-OH (Figure 3). The residues released were exactly those predicted from the composition of tryptic peptide T-8 and since this digestion was performed with whole amyloid protein A, it confirms the placement of tryptic peptide T-8 at the carboxyl terminus of the molecule.

Discussion

The present data indicate that monkey amyloid protein A contains a single polypeptide chain of 76 amino acid residues. The protein is homogeneous except that approximately 22% of the molecules lack the amino-terminal arginine residue (Benditt *et al.*, 1971). Treatment with cyanogen bromide produced the expected three fragments whose aggregate amino acid composition agreed with that of the starting material. Thus no contaminating protein or peptide containing a blocked amino-terminal residue was present in the preparation.

The two methionyl residues were suitably disposed to permit sequenator analysis to be extended through 70 of the 76 residues. The remaining six carboxyl-terminal residues were placed by manual Edman degradation of the carboxyl-terminal tryptic peptide and by digestion of the whole protein with yeast protease C. No other carboxypeptidase pure enough for sequence determination would have been effective at the low pH necessary to solubilize amyloid protein A, and none

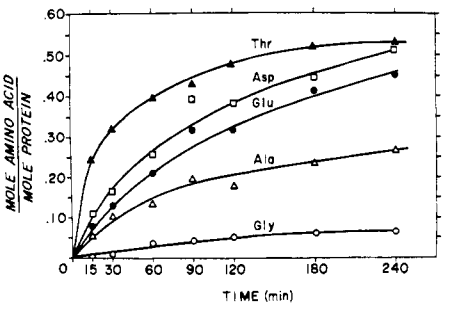

FIGURE 3: Time course of the release of amino acid residues from monkey amyloid protein A by digestion with yeast protease C.

would have sequentially liberated the acidic residues as effectively as did yeast protease C.

Amyloid protein A has now been found in several human patients (Benditt and Eriksen, 1972a) and in monkeys (Benditt and Eriksen, 1972b) with various inflammatory diseases. A high degree of homology between the amyloid A proteins from monkey and man has been demonstrated (Benditt *et al.*, 1971). Recently a similar protein has been extracted from the liver of Pekin ducks. Within the expected limits of phylogenetic variation, this protein is homologous to the amyloid A proteins derived from the two other species (L. H. Ericsson, N. Eriksen, and E. P. Benditt, in preparation).

Recently Ein *et al.* (1972) presented evidence that 15 amino-terminal residues of a major constituent of an amyloid substance derived from the spleen of a patient with a chronic inflammatory disease (rheumatoid arthritis) are identical with the corresponding sequence of amyloid protein A. The protein reported by Ein *et al.* had an estimated molecular weight of 5000 and lacked proline and valine. The primate amyloid protein A reported here has a molecular weight of 8621. Since the monkey and the human proteins are so closely homologous and since the single proline and valine residues in the monkey amyloid protein A are carboxyl terminal to residue 48, it may be anticipated that the peptide of Ein *et al.* represents a fragment of amyloid protein A lacking the carboxyl-terminal portion.

Amyloid proteins characterized by the amino acid composition, amino acid sequence, and size described herein all have been derived from tissues of patients and animals with type A amyloidosis. The proteins present in type B amyloidosis, either without obvious disease or associated with neoplasms, are strikingly different (Benditt and Eriksen, 1971). In other instances proteins have been recovered which have amino-terminal sequences that are related to the variable portions of the light chains of human immunoglobulin (Glenner *et al.*, 1971). Clearly there is no structural relationship between these latter proteins and the amyloid protein of the A type.

Amyloid protein A bears no homologous relationship to any protein or peptide of known function. Today it is not known whether the amyloid protein A is itself a normal cellular constituent or a fragment of a functional protein; nor is it known where the protein is synthesized. It is not detectable in normal tissue by chemical criteria (Benditt and Eriksen, 1971) but these criteria may not be sensitive enough to detect the protein in nondiseased tissue. Thus the determination of the physiological role of amyloid protein A and the site of its cellular synthesis await further investigation.

Acknowledgments

The authors are indebted to Lowell H. Ericsson for his assistance in the sequenator analyses, to Richard R. Granberg for the amino acid analyses, and to Dr. Koiti Titani for his advice.

Addendum

After this paper was submitted for publication, Franklin *et al.* (1972) reported the isolation of amyloid proteins from two human patients. The amino-terminal sequences (up to residues 21–33) show a high degree of homology to the present sequence of the monkey protein and are identical with the amino-terminal 24 residues of the human protein reported by us (Benditt *et al.*, 1971).

References

Benditt, E. P., and Eriksen, N. (1971), *Amer. J. Pathol. 65*, 231.

Benditt, E. P., and Eriksen, N. (1972a), *Lab. Invest. 26*, 615.

Benditt, E. P., and Eriksen, N. (1972b), *Acta Pathol. Microbiol. Scand.* (in press).

Benditt, E. P., Eriksen, N., Hermodson, M. A., and Ericsson, L. H. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 19*, 169.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.

Ein, D., Kimura, S., and Glenner, G. G. (1972), *Biochem. Biophys. Res. Commun. 46*, 498.

Franklin, E. C., Press, M., Levin, M., and Frangione, B. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22*, 121.

Glenner, G. G., Terry, W., Harada, M., Isersky, C., and Page, D. (1971), *Science 172*, 1150.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry* (in press).

Kuhn, R. W., Walsh, K. A., and Neurath, H. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 877.

Liu, T.-Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246*, 2842.

Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244*, 5597.