Takemori, S., Nakazawa, K., Nakai, Y., Suzuki, K., & Katagiri, M. (1968) *J. Biol. Chem. 243*, 313.
Takemori, S., Tajima, H., Kawahara, F., Nakai, Y., & Katagiri, M. (1974) *Arch. Biochem. Biophys. 160*, 289.
Tsou, C.-L. (1962) *Sci. Sin. (Engl. Ed.) 11*, 1535.
Walsh, C. (1978) *Annu. Rev. Biochem. 47*, 881.
Walsh, C. (1980) *Acc. Chem. Res. 13*, 148.
Walsh, C. T., Schonbrunn, A., & Abeles, R. H. (1971) *J. Biol. Chem. 246*, 6855.
Walsh, C. T., Schonbrunn, A., Lockridge, O., Massey, V., & Abeles, R. H. (1972) *J. Biol. Chem. 247*, 6004.
Werber, M. W., Moldovan, M., & Sokolovsky, M. (1975) *Eur. J. Biochem. 53*, 207.
Yang, P. C., & Schwert, G. W. (1972) *Biochemistry 11*, 2218.

Quantitative Solid-Phase Edman Degradation for Evaluation of Extended Solid-Phase Peptide Synthesis†

Gary R. Matsueda, Edgar Haber,* and Michael N. Margolies

ABSTRACT: Quantitative solid-phase Edman degradation was used for the amino acid sequence analysis of synthetic peptidyl-resins prepared by the Merrifield solid-phase procedure. A model peptide, Ala-[^{3}H]Pro-Ala-Gly-Phe-Ala-Gly-, was synthesized on a solid support and was sequenced to measure the efficiency of the solid-phase sequencing protocol used. An average of 92% of the first four residues was removed from the peptidyl-resin as indicated by subtractive amino acid analysis. Quantitation of the radioactive proline residue at cycle 2 revealed that it was efficiently recovered both from the acid conversion procedure (99%) and also following high-pressure liquid chromatography of the phenylthiohydantoin (Pth) amino acid (88%). In order to facilitate identification and quantification of the side chain protected Pth amino acids, we prepared these derivatives and characterized them by high-pressure liquid chromatography. Thereafter, by use of solid-phase Edman degradation as an analytical procedure, the synthesis of residues 2–118 of the heavy-chain variable region (V_H) of a homogeneous rabbit antibody was undertaken. At 10–15-residue intervals during the solid-phase synthesis, samples of peptidyl-resin were removed from the synthesis vessel and sequenced. When gross synthetic errors caused by deletion of amino acid residues were detected, the solid-phase synthesis was terminated and restarted by using modified protocols. A 117-residue peptidyl-resin was prepared finally which possessed the desired amino acid sequence as indicated by a series of solid-phase Edman degradation experiments. In the final degradation experiment on the 117-residue peptidyl-resin, a 92% efficiency for the automatic Edman reaction was measured ([^{3}H]Leu, penultimate amino-terminal residue). We have found two advantages for the concurrent use of solid-phase Edman degradation during an extended solid-phase synthesis: (1) on the basis of the levels of error due to incomplete incorporation of amino acids, the solid-phase assembly could be terminated in favor of restarting the synthesis, hence avoiding further work on a defective product and (2) direct verification of incorporation of amino acids, which during acid hydrolysis are destroyed (Cys, Trp) or are deamidated (Asn, Gln), is possible by high-pressure liquid chromatography of the corresonding Pth derivatives.

The solid-phase method of peptide synthesis as introduced by Merrifield (1963) has enjoyed widespread use as evidenced by an increasing number of reports describing the synthesis of biologically active peptides [reviewed by Erickson & Merrifield (1976)]. The basic solid-phase concept has been extended to the synthesis of oligosaccharides (Frecht & Scheurch, 1971) and oligonucleotides (Chapman & Kleid, 1973). In solid-phase peptide synthesis, improvements of procedure, protected amino acid derivatives, and solid supports have facilitated the synthesis of well-characterized peptides containing up to 40 amino acid residues (Yamashiro & Li, 1973; Tregear et al., 1974; van Rietschoten et al., 1975; Coy et al., 1977). Briefly, this has been accomplished in the following way. The carboxy-terminal amino acid is first attached covalently to a solid-support, usually polystyrene beads. This is followed by the sequential coupling of protected amino acids to the resin-bound amino groups until the desired peptide has been assembled. The completely deprotected peptide is then obtained by treatment of the peptidyl-resin with a cleavage reagent, usually liquid hydrogen fluoride. The crude product must then be purified and characterized with respect to its chemical homogeneity before any biological activity can be measured.

In order to establish the structure–activity relationships among analogues of peptides containing more than 40 residues, it is necessary to improve methods of chemical synthesis. For the solid-phase synthesis of a long peptide, it is anticipated that improved analytical methods will lead to improved synthetic methods. In this report we evaluate solid-phase Edman degradation as one analytical method for extended solid-phase peptide synthesis. Our goals were to verify experimentally the sequence of the peptide which is being synthesized and to detect error sequences which arise during synthesis from either incomplete coupling or incomplete deprotection.

It has been recognized that the Edman degradation (Edman, 1957) which is used to determine the amino acid sequence of peptides should prove useful in evaluating the progress of the solid-phase synthesis (Niall et al., 1972; Fankhauser et al., 1974; Birr & Frank, 1975). However, early attempts were hampered by difficulties in adapting the degradative procedure for amino acid sequencing of synthetic peptidyl-resins (Niall et al., 1972) and, in particular, in quantifying the resulting

† From the Cellular and Molecular Research Laboratory, Departments of Pathology, Medicine and Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114. *Received July 23, 1980.* Supported by National Institutes of Health Grant HL-19259 and a grant from Reynolds Industries.

0006-2960/81/0420-2571$01.25/0

phenylthiohydantoin (Pth)[1] amino acids (Fankhauser et al., 1974). Subsequent developments including solid-phase sequencing introduced by Laursen (1971), the advent of high-pressure liquid chromatography (LC) quantification of Pth amino acids (de Vries et al., 1975; Zimmerman et al., 1977), and the recognition of the lability of the peptide to resin linkage during sequencing (Birr & Frank, 1975; Matsueda & Margolies, 1979) have facilitated a routine utilization of solid-phase Edman degradation for the evaluation of solid-phase Merrifield synthesis.

Materials and Methods

Reagents. *tert*-Butoxycarbonyl (Boc) amino acids, purchased from Peninsula Laboratories or Bachem, were of the L configuration except for glycine. Analytical grades of reagents and solvents were used directly with the following exceptions: trifluoroacetic (F_3CCOOH) from Halocarbon was distilled (bp 89–90 °C) and stored at 4 °C until used; triethylamine (TEA) from J. T. Baker Chemical Co., was distilled from ninhydrin and stored at 4 °C; technical grade CH_2Cl_2 from Dow Chemical Co. (factory-sealed drum) was distilled from CaH_2. Reagent grade *N*,*N*-dimethylformamide (DMF) was stored at least 48 h over Linde 4-Å molecular sieves before use. Resin beads, copoly(styrene–1% divinylbenzene), 200–400 mesh, from Lab Systems were passed through a 149-μm mesh screen to remove amorphous particles. Sequencer reagents except for F_3CCOOH were purchased from Beckman Instruments or Pierce Chemical Co.

General Methods. Hydrolysis of peptidyl-resin samples in propionic acid–HCl (1:1) was performed in vacuo according to the procedure of Westall et al. (1972), except that the heating time at 130 °C was 4 h instead of 2 h. The amino acid analyses were performed on a Durrum analyzer, Model D-500. The Sequemat, Model 12L, was used for automatic solid-phase Edman degradation using the program described by Laursen et al. (1975) except for the addition of a 2-min methanol wash prior to F_3CCOOH. Synthetic peptidyl-resin samples were used directly for sequencing experiments. The sample resins (5–10 mg) were mixed with ~1 cm^3 of glass beads, 200–400 mesh. An even dispersion of the resin sample was obtained by tumbling the Sequemat reaction column during the packing operation. This process most effectively avoided the precolumn leakage of reagents and solvents which was occasionally a problem during automatic operation due to excessively high column back-pressure.

Identification of Pth Amino Acids. At the completion of the preselected number of Edman degradation cycles, the contents of the tubes containing the anilinothiazolinone (Atz) amino acids were dried under a stream of N_2 at 40 °C. This was followed by the manual conversion of the Atz amino acids to the corresponding Pth amino acids (1 N HCl, 10 or 13 min at 80 °C). After ethyl acetate extraction of the Pth amino acids and N_2 drying of the ethyl acetate solution, Pth-Nle was added as an internal reference marker. Pth amino acids, which were dissolved in acetonitrile, were quantified by LC (Margolies & Brauer, 1978) on a Waters liquid chromatograph, using a 4 mm × 30 cm Microbondapak C_{18} column (Waters Associates) at room temperature. The program utilized gradient curve 7 of the solvent programmer (Waters Model 660), beginning with 34% solvent B and ending with 55% solvent B, over 15 min at 1.8 mL/min. At the end of the gradient, isocratic elution continued at 55% solvent B. Solvent A was composed of 10% acetonitrile in 0.01 M sodium acetate buffer (pH 4.08); solvent B was composed of 90% acetonitrile in 0.01 M sodium acetate buffer (pH 6.0). The elution of Pth amino acids was detected by absorbance at 254 nm and quantified by using a Hewlett-Packard integrator, Model 3385.

Synthesis of Model Peptidyl-resin, Ala-[^{3}H]Pro-Phe-Ala-Phe-Ala-Gly-Pam(AcAbu)-resin, for Evaluation of Sequencing Efficiency. The preparation of hydroxymethylphenylacetamidomethyl(*N*-acetylaminobutyramidomethyl)-resin has been described (Matsueda & Haber, 1980). This resin retained the acid resistance of the phenylacetamidomethyl (Pam) group as described by Mitchell et al. (1976), but, in addition an N-acetylated residue of α-aminobutyric acid (Abu) was incorporated to serve as an internal reference amino acid which could be measured by amino acid analysis following propionic acid–HCl hydrolysis (Matsueda & Haber, 1980). Boc-Gly was coupled to this resin by using dicyclohexylcarbodiimide (DCC) and 4-(*N*,*N*-dimethylamino)pyridine in 10 mL of CH_2Cl_2, and the benzoylation was performed according to Chang et al. (1976). The remaining amino acids as Boc derivatives were coupled sequentially by using standard manual procedures (Stewart & Young, 1969). Boc-[^{3}H]Pro ([^{3}H]Pro, New England Nuclear) was prepared by using procedure A of Moroder et al. (1976). The resulting peptidyl-resin, Ala-[^{3}H]Pro-Phe-Ala-Phe-Ala-Gly-Pam(AcAbu) resin, was used in the sequence study described below.

This model ^{3}H-labeled peptide (5 mg) was sequenced automatically for four cycles; the waste effluents from each Edman degradation cycle were collected separately. The waste fractions were dried with N_2 and the residues dissolved in MeOH–H_2O (1:1) prior to liquid scintillation counting (Liquiscint, National Diagnostics, 10 mL). At the completion of the sequencing experiment, the resin samples along with the glass beads were transferred directly to a hydrolysis tube with methanol. After the evaporation of methanol, the resin was hydrolyzed with propionic acid–HCl (1:1) prior to amino acid analysis. The recovery of Pth-[^{3}H]Pro upon LC analysis was measured by counting an aliquot of the LC effluent (0.5 mL/fraction).

Synthesis of Peptidyl-resins for Solid-Phase Preparation of Side Chain Protected Pth Amino Acids. In order to identify certain Pth amino acid derivatives by LC, it was necessary to synthesize a series of peptidyl-resins which contained the various side chain protected amino acids. The sequence framework Ala-X-Leu-Y-Ala- was used in which X and Y corresponded to two different protected amino acids. The synthesis of five framework peptides was required to accommodate the following ten amino acids: Asp(OBzl), Glu(OBzl), Ser(OBzl), Thr(OBzl), Tyr(O-2,6-Cl_2Bzl), Trp(CHO), Cys(Acm), Lys(2-ClZ), Arg(Tos), and Tyr(2-BrZ).

An example of one synthesis follows: Starting with 1 g of NH_2CH_2 resin (0.89 mmol of amino groups/g of resin), Ala-Asp(OBzl)-Leu-Thr(OBzl)-Ala-Gly-$NHCH_2$-resin was prepared by using a Beckman synthesizer, Model 990B, for the sequential coupling of Boc-Gly, Boc-Ala, Boc-Thr(OBzl), Boc-Leu, Boc-Asp(OBzl), and finally Boc-Ala. The following synthetic protocol was used for a single coupling cycle: (1) wash with CH_2Cl_2 (all volumes 40 mL) 3 times for 1.5 min; (2) neutralize with TEA–CH_2Cl_2 (1:9), 2 × 1.5 min and then 1 × 5 min; (3) wash with CH_2Cl_2, 6 × 1.5 min; (4) couple with Boc amino acid–dicyclohexylcarbodiimide (DCC) (1:1),

[1] Abbreviations used: Pth, phenylthiohydantoin; LC, high-pressure liquid chromatography; Abu, α-aminobutyric acid; Tos, tosyl; Pam, phenylacetamidomethyl; Aoc, *tert*-amyloxycarbonyl; Boc, *tert*-butoxycarbonyl; TEA, triethylamine; F_3CCOOH, trifluoroacetic acid; DCC, *N*,*N*′-dicyclohexylcarbodiimide; Atz, anilinothiazolinone; Bzl, benzyl; DMF, *N*,*N*-dimethylformamide; Acm, acetamidomethyl; Z, benzyloxycarbonyl.

Table I: Amino Acid Composition of Hexapeptidyl-resins Used for Preparation and LC Characterization of Side Chain Protected Pth Amino Acids

	sequence of model peptide: Ala-X-Leu-Y-Ala-Gly-$NHCH_2$-resin		amino acid composition[a]				
	X position	Y position	X	Leu	Y	Ala	Gly
A	Asp(OBzl)	Thr(OBzl)		0.89	0.82	1.07	1.22
			0.89	1.01	0.85	1.92	1.35
B	Ser(OBzl)	Glu(OBzl)		0.83	0.97	1.02	1.18
			0.51	0.99	1.09	2.02	1.37
C	Cys(Acm)	Tyr(O-2,6-Cl_2Bzl)		0.93	0.91	1.01	1.15
			+[b]	0.93	1.05	1.81	1.20
D	Trp(CHO)	Arg(Tos)		0.93	1.02	1.01	1.03
			+[b]	0.97	1.07	1.86	1.11
E	Tyr(2-BrZ)	Lys(2-ClZ)		1.03	0.98	0.98	1.01
			0.95	1.05	0.96	2.00	1.06

[a] Amino acid analyses were done after the addition of four and after the addition of six amino acids to the resin, to prove the correct position of the side chain protected amino acids at the X and Y positions. [b] Extensively degraded during acid hydrolysis.

2 mmol each in 15 mL of CH_2Cl_2 for 120 min; (5) wash with CH_2Cl_2, 6 × 1.5 min; (6) deprotect with F_3CCOOH–CH_2Cl_2 (1:3), 2 × 1.5 min and then 1 × 30 min; (7) wash with CH_2Cl_2, 6 × 1.5 min. The completeness of coupling was monitored by using the ninhydrin test (Kaiser et al., 1970). If coupling was incomplete, the same Boc amino acid was recoupled by repeating steps 1–5. These steps were followed by a repetition of steps 1–3 and then (8) acetylation with 10 mmol of acetic anhydride–TEA (1:1) in 15 mL of CH_2Cl_2.

Four other synthetic peptidyl-resins containing the same framework sequence were synthesized as described above. Each contained two different protected amino acids as specified in Table I. For the coupling of Boc-Trp(CHO), Boc-Cys(Acm), and Aoc-Arg(Tos), 10% DMF in CH_2Cl_2 was used as the solvent.

Amino acid analysis of the tetrapeptide intermediate was used to identify the side-chain amino acid placed in the Y position (i.e., Leu-Y-Ala-Gly-resin). These data, along with the amino acid composition of the completed hexapeptidyl-resins are shown in Table I.

In turn, each of the five peptidyl-resins was sequenced as described above, in order to provide the desired Pth amino acids with side chain protected functional groups. These Pth amino acid derivatives were subsequently characterized by LC, as described above.

Solid-Phase Synthesis of Immunoglobulin Fragment 3374 Peptidyl-resins. Brief descriptions of three attempts to synthesize the variable region of a heavy chain (V_H) of a rabbit anti-type III pneumococcal polysaccharide antibody follow. The amino acid sequence of this peptide as proposed by Margolies & Haber (1978) is shown in Figure 1. Experimental details are presented to illustrate the various synthetic tactics employed in order to correlate the effectiveness of the synthetic protocols with the results of the solid-phase peptide sequencing experiments. The same coupling protocols for Boc-Asn and Boc-Gln were used in each of the three syntheses. For the neutralization of resin, a 0.2 M solution of Boc-aminoacyl-*p*-nitrophenyl ester in DMF was added and stirred for 120 min.

(1) Preparation of Peptidyl-resin A, Corresponding to V_H-3374 (Residues 84–118). Aoc-Arg(Tos)-Pam(AcAbu)-resin was prepared by coupling AocArg(Tos) to $HOCH_2$-Pam(AcAbu)-resin (Matsueda & Haber, 1980) using DCC and 4-(dimethylamino)pyridine for 2 h as described by Chang et al. (1976). Following the prescribed benzoylation, the resulting amino acyl resin was treated with F_3CCOOH–CH_2Cl_2 (1:3), 1 × 1.5 and 1 × 30 min, in order to remove the Aoc group. Amino acid analysis indicated a substitution of 0.07 mmol of Arg/g of resin.

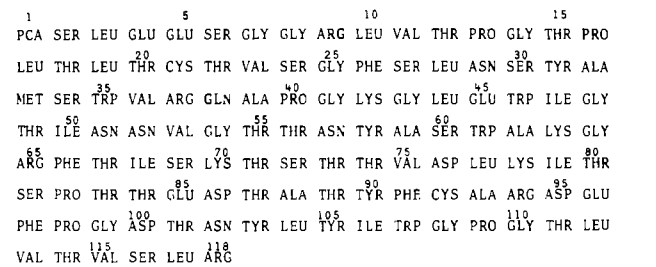

FIGURE 1: Amino acid sequence of variable region domain (1–118) from the heavy chain of a homogeneous rabbit anti-type III pneumococcal antibody 3374 (Margolies & Haber, 1978).

For the synthesis of peptidyl-resin A which corresponded to V_H-3374 (residues 84–118), 4.8 g of Arg(Tos)-Pam(AcAbu)-resin was placed in the reaction vessel of a Beckman synthesizer, Model 990B. The CH_2Cl_2-swollen volume of the resin was ~35 mL. All wash volumes were 80 mL. Deprotection was obtained by the following procedure: (a) 3 × 2 min with CH_2Cl_2 containing 1 mg/mL indole; (b) 2 × 2 and then 1 × 30 min with F_3CCOOH–CH_2Cl_2 (1:3) containing 1 mg/mL indole; (c) 3 × 2 min with CH_2Cl_2 containing 1 mg/mL indole. Neutralization was performed by the following procedure: (a) 3 × 2 min with CH_2Cl_2; (b) 2 × 2 and then 1 × 5 min with TEA–CH_2Cl_2 (1:9). Coupling was performed by the following: (a) 3 × 2 min with CH_2Cl_2; (b) 1 mmol each of Boc amino acid–DCC (1:1) in 15 mL for 15 min and then after 60 min, addition of 50 mL of CH_2Cl_2; (c) 3 × 2 min with CH_2Cl_2. Recoupling of each residue was performed automatically by the following procedure: first neutralization of the resin as described above and then (a) 3 × 2 min with CH_2Cl_2; (b) Boc amino acid–DCC (2:1) in 15 mL for 15 min with 2 mmol of Boc amino acid and then after 60 min, addition of 50 mL of CH_2Cl_2; (c) 3 × 2 min with CH_2Cl_2. Ninhydrin monitoring was not performed. This protocol resembles many "standard" DCC coupling protocols (Stewart & Young, 1969). After the coupling of Trp^{107} and Ala^{93} peptidyl-resin samples were taken from the synthesizer reaction vessel for solid-phase sequencing experiments.

(2) Preparation of Peptidyl-resin B, Corresponding to V_H-3374 (Residues 76–118). The aminoacyl resin was prepared as follows: 15 g of $HOCH_2$Pam(AcAbu) resin (Matsueda & Haber, 1980) was placed in the large (500 mL) reaction vessel of the Beckman synthesizer and treated with Aoc-Arg(Tos), 4-(dimethylamino)pyridine, and DCC, 7.5 mmol, each for 45 min. After being washed with 100 mL of CH_2Cl_2, 6 × 2 min, the resin was treated with fresh reagents for 75 min and then benzoylated. The substitution of Arg(Tos) was found to be 0.19 mmol/g of resin.

For the synthesis of a peptidyl-resin B corresponding to V_H-3374 (residues 71–118), 2 g of the above aminoacyl-resin was placed in the 250-mL reaction vessel of the Beckman synthesizer, model 990B. The volume neutralization was performed as described for the synthesis of peptidyl-resin A. The coupling program was modified by using 2 mmol of Boc amino acid and 2 mmol of DCC in 16 mL of CH_2Cl_2. Recoupling was also modified by using 4 mmol of Boc amino acid and 2 mmol of DCC in 22 mL of CH_2Cl_2 which were mixed in situ along with the neutralized resin. The completeness of coupling was monitored with the ninhydrin reagent (Kaiser et al., 1976). Recoupling in CH_2Cl_2 or in DMF was performed if incomplete coupling was detected. If the ninhydrin test was still positive after recoupling, acetylation of the neutralized

Table II: Efficiency of the Solid-Phase Edman Degradation of Ala-[^{3}H]Pro-Phe-Ala-Phe-Ala-Gly-Pam(AcAbu)-resin:[a] Amino Acid Composition of Peptidyl-resin Samples[b]

amino acid	before sequencing		after sequencing[c]		difference		Edman degradation efficiency/cycle (%)
	expected	found	expected	found	expected	found	
Ala	3	2.96	1	1.28	2	1.68	84
Pro	1	0.99	0	0.00	1	0.99	99
Phe	2	1.93	1	0.93	1	1.00	100
Gly	1	1.02	1	1.06	0	−0.04	
							mean: 92

[a] Efficiency refers to the removal of amino acids from the peptidyl-resin as determined by subtractive amino acid analysis. [b] Peptidyl-resin samples were sealed in vacuo with propionic acid–HCl (1:1) and heated for 4 h at 130 °C, before amino acid analysis. Abu served as internal reference amino acid. [c] Solid-phase Edman degradation was performed automatically. After four degradative cycles, the resin was collected and hydrolyzed.

resin was performed by using 0.05 M acetic anhydride in CH_2Cl_2 for 20 min. Following the coupling of Tyr^{105}, Tyr^{90}, and Asp^{76}, peptidyl-resin samples were taken from the synthesizer reaction vessel and subjected to solid-phase sequencing experiments.

(3) Preparation of Peptidyl-resin C, Corresponding to V_H-3374 (Residues 2–118). This synthesis began with 2.0 g of Aoc-Arg(Tos) resin which was prepared as described for peptidyl-resin B. The synthetic protocol was modified in several ways as compared to the protocol used for peptidyl-resin B. For deprotection, two acidic reagents were used, neither of which contained indole as a scavenger. After the resin was washed with 45 mL of CH_2Cl_2, 6 × 1.5 min, F_3C-COOH–CH_2Cl_2 (1:3) was added, 2 × 1.5 min and then 1 × 5 min, and then CH_2Cl_2 was added, 6 × 1.5 min. The coupling program was modified to use preformed symmetric anhydride (2 mmol/20 mL of CH_2Cl_2) which was added to a neutralized resin which had been treated with hexane 5 times without stirring. After 20 min, 0.25 mmol of TEA in toluene was added, and the stirring was continued for another 20 min. The symmetric anhydrides were formed at 0 °C for 20 min before filtration of the dicyclohexylurea and delivery to the synthesizer reaction vessel (Hagenmaier & Frank, 1972; Yamashiro & Li, 1974).

The recoupling program was identical with the coupling program except for the omission of the TEA addition. Recoupling was performed routinely before any ninhydrin monitoring was attempted. When additional recoupling failed to yield a negative ninhydrin test, acetylation was performed by using the coupling program, but substituting 10 mmol of acetic anhydride for the 2 mmol of symmetric anhydride. Following the coupling of residues Thr^{111}, Thr^{101}, Tyr^{90}, Asp^{76}, Lys^{63}, Asn^{52}, Pro^{40}, Ser^{30}, Thr^{20}, Leu^{10}, and Ser^{2}, samples of peptidyl-resin were taken from the synthesizer reaction vessel for solid-phase sequencing experiments.

Results

The utility of solid-phase Edman degradation for the evaluation of solid-phase peptide synthesis was demonstrated by two series of experiments. In the first set of experiments the significance of LC quantification of Pth amino acids generated by solid-phase sequencing was examined. These experiments were designed to determine whether the LC-generated data represented quantitatively the synthetic peptides which were anchored originally to the resin. In the second set of experiments, solid-phase Edman degradation was used as an analytical procedure for the improvement of sequence uniformity of the peptidyl-resin intermediates. For these experiments the preparation and LC characterization of side chain protected Pth amino acids was necessary. By use of this information, amino acid sequence analysis of protected peptidyl-resin samples was possible. The utility of quantitative solid-phase Edman degradation was demonstrated during extended solid-phase synthesis of a variable region domain (V_H) from the anti-type III pneumococcal antibody 3374.

Synthesis and Solid-Phase Edman Degradation of Model ^{3}H-Labeled Peptide. The solid-phase synthesis of Ala-[^{3}H]-Pro-Phe-Ala-Phe-Ala-Gly-OCH_2-Pam(AcAbu)-resin proceeded without difficulty. A single DCC-mediated coupling was sufficient for the complete incorporation of each amino acyl residue as evidenced by the ninhydrin test. The amino acid analysis of the hydrolysate of the peptidyl-resin was satisfactory as shown in Table II. The specific radioactivity of the peptide was 0.08 mCi of ^{3}H/mmol of peptide.

A sample of ^{3}H-labeled peptidyl-resin was subjected to four cycles of automatic Edman degradation. Several aspects of this experiment were studied: (1) the efficiency of the Edman degradation by subtractive amino acid analysis, (2) the distribution of the ^{3}H-labeled residue among the fractions which were collected, and (3) the recovery of [^{3}H]proline during LC quantification.

Following the automatic solid-phase sequencing experiment, the resin, including glass beads, was collected and treated with propionic acid–HCl. The amino acid analysis of the hydrolysate is compared in Table II with the amino acid composition of the peptidyl-resin prior to sequencing. On the basis of a subtractive analysis, the average efficiency of the four Edman degradation cycles was 92%. [^{3}H]Proline and phenylalanine at cycles 2 and 3, respectively, were almost quantitatively removed by the degradation experiment. This provided evidence that the model peptide chains which were attached to the solid support were accessible to sequencing reagents. This result was essential for a meaningful sequence analysis of a synthetic peptidyl-resin sample.

By taking advantage of the ^{3}H-labeled proline residue, it was possible to measure proline without regard to its chemical form. During each cycle of the automatic degradation, the waste fractions were pooled and dried with N_2, as well as the fraction which was expected to contain the Atz-derivatized amino acids. Following aqueous HCl conversion of the Atz amino acid fraction and extraction with ethyl acetate, both aqueous and ethyl acetate fractions were saved. The distribution of radioactivity in all fractions is presented in Table III. Most (95%) of the total radioactivity recovered was found in various fractions of cycle 2. An additional 3% was found in cycle 3. A "carryover", in this instance 3%, is commonly observed in liquid-phase Edman degradation for natural peptides as well as synthetic peptides. This is attributed usually to an incomplete coupling and/or cleavage during Edman degradation of an amino acid residue.

Another aspect of the sequencing experiment, which was usually observed for synthetic peptides, was seen in cycle 1,

Table III: Solid-Phase Edman Degradation of Ala-[^{3}H]Pro-Phe-Ala-Phe-Ala-Gly-Pam(AcAbu)-resin

degradation cycle	amino acid expected	Pth amino acid[a] found in organic fraction (nmol)	cpm due[b] to [^{3}H]Pro	total radioactivity recovered (%)	distribution of total radioactivity (%)		
					pooled waste fractions	fractions after conversion: aqueous layer	fractions after conversion: ethyl acetate layer
1	Ala	Ala (1035) Pro (2.7)	350	1.0	0.1	0.1	0.8
2	Pro	Pro (361) Ala (48) Phe (12)	32130	95.0	0.1	0.9	94.0
3	Phe	Phe (581) Pro (17) Ala (25)	1150	3.5	0.1	0.1	3.3
4	Ala	Ala (796) Phe (36) Pro (2.6)	160	0.5	0.0	0.0	0.5

[a] Quantified by detection of the Pth amino acids at 254 nm following LC separation using a C_{18} Microbondapak column and gradient elution with acetonitrile. [b] Aliquots of samples for liquid scintillation counting were first dried in glass vials under a N_2 stream, then dissolved in methanol, and finally mixed with 10 mL of Liquiscint prior to liquid scintillation counting. Numbers are corrected for background.

where 1% of the total radioactivity was found. The [^{3}H]proline detected in this cycle was not expected until the second cycle, in which most of the [^{3}H]proline (94%) was found. The observation of a Pth amino acid in an Edman degradation cycle before it is expected has been referred to as "preview" (Niall et al., 1972) of the amino acid sequence and is evidence for a synthetic error. This observation is explicable by the failure to completely incorporate an amino acyl residue. There are two possible causes for the deletion of an amino acid in a fraction of the peptide chains attached to the resin. First, incomplete coupling of Boc-Ala to the penultimate prolyl residue will result in a fraction of the peptide chains in which the amino-terminal residue remains proline. Another cause for the deletion of an amino acyl residue during synthesis is the failure to completely deprotect the *N*-α-Boc of the prolyl residue. This would result in a fraction of peptide amino groups which is unavailable for the coupling reaction of Boc-Ala. Thus, following the deprotection step in the next synthetic cycle, alanine is found at the amino position, but, in addition, there is also a small amount of proline. These results, which were obtained by liquid scintillation measurements of [^{3}H]proline, were confirmed by the LC analysis as shown in Table III.

The recovery of radioactivity due to [^{3}H]proline was measured during LC analysis. Following acid conversion, the ethyl acetate layer from cycle 2 was used because it accounted for 94% of the total radioactivity recovered from the solid-phase Edman degradation (Table III). An aliquot was injected into the liquid chromatograph, and the effluent fractions were collected. In Figure 2, the profiles of radioactivity measured and the absorbance at 254 nm are shown. The recovery of the radioactivity associated with peaks A and B was 88%. Peak B coincided with an authentic Pth-Pro standard. Peak A, which was larger, was designated Atz-Pro. This conclusion was based on the following observations: (1) nearly all of the observed absorbance elutes in peak A when an unconverted sample from the sequencer was injected; (2) with increasing time used for conversion, peak B (Pth-Pro) enlarges while peak A recedes. When another identical aliquot was injected with the final conditions (55% solvent B) attained by the gradient programmer, 92% of the radioactivity was recovered as a single peak.

These experiments establish that, for the above model system, solid-phase Edman degradation was useful as an analytical procedure. The solid-phase sequence analysis provided quantitative data which could be used with confidence

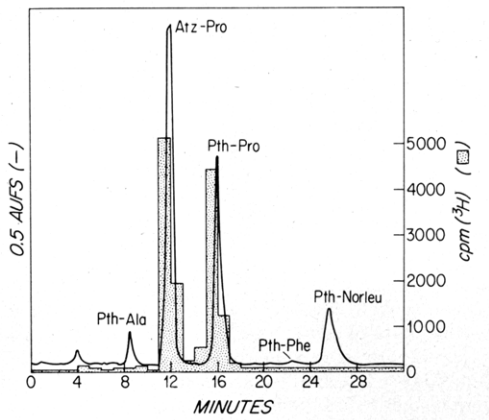

FIGURE 2: High-pressure liquid chromatography of the Pth amino acid from the second cycle of automatic Edman degradation of Ala-[^{3}H]Pro-Phe-Ala-Phe-Ala-Gly-resin. The designation of Atz-Pro was based upon a separate experiment in which increasing conversion time led to a decrease in the peak size of Atz-Pro with a corresponding increase of Pth-Pro. Pth-Nle was used as an internal reference marker for the LC analysis. In this particular cycle, the detection of Pth-Ala was due to the sequencing overlap and Pth-Phe to preview.

for the analysis of solid-phase synthesis. In addition to an efficient extraction and recovery following LC analysis, these experiments demonstrate that all of the synthetic peptides which were attached to the resin were degraded (Table II), thus providing a representative sample of the amino-terminal amino acid at each degradative cycle.

LC Characterization of Pth Amino Acids Containing Side Chain Protecting Groups. For characterization of the side chain protected Pth amino acids by LC, five reference peptidyl-resins were synthesized. The amino acid composition of these reference hexapeptidyl-resins is shown in Table I. In addition to the amino acid composition of each completed peptide, amino acid analysis was performed on the intermediate tetrapeptidyl resin. From these data, the assignment of the protected amino acids (X and Y) within the sequence framework, Ala-X-Leu-Y-Ala-Gly-, was unequivocal. This peptide sequence was chosen because it permitted the calculation of a repetitive yield, which was based upon LC quantification of Pth-Ala at steps 1 and 5. By use of these values of Pth-Ala the repetitive yield (RY) value was calculated according to

$$\mathrm{RY} = \frac{\text{nanomoles of Pth-Ala at step } j^{1/(j-i)}}{\text{nanomoles of Pth-Ala at step } i}$$

Table IV: Identification of Phenylthiohydantoin Derivatives of Side Chain Protected Amino Acids by High-Pressure Liquid Chromatography[a]

amino acid	side chain protecting group	elution time (min)	area[b]	std Pth amino acids	elution time (min)
Asp	*O*-benzyl	2.29[c]	0.030	Asp	2.27
		15.96	0.281	Glu	3.11
		19.13	0.294	Gly	3.54
Glu	*O*-benzyl	3.13[c]	0.146	Ala	4.64
		17.95	0.587	Tyr	5.45
		19.85	0.012	Pro	8.62
Thr	*O*-benzyl	6.80	0.024	Val	9.02
			(0.174[d])	Trp	11.61
		16.35	0.106	Phe	11.61
		21.10	0.379	Ile	12.21
Ser	*O*-benzyl	11.20	0.015	Leu	13.09
		14.89	0.102	Nle	13.53
		17.34	0.249		
		19.23	0.036		
Cys	*S*-acetamido-methyl	3.38	0.592		
		4.02	0.020		
Tyr	*O*-2,6-dichloro-benzyl	19.41	0.035		
		25.10	0.295		
	O-2-bromobenzyl-oxycarbonyl	25.53	0.162		
		32.99	0.549		
Lys	*N*-2-chlorobenzyl-oxycarbonyl	19.47	0.203		
		21.32	0.430		
Arg	*N*-tosyl	8.77	0.631		
		10.61	0.138		
Trp	*N*-formyl	11.55[c]	0.103		
		12.70	0.212		
		16.51	0.079		
		17.41	1.269		

[a] Pth amino acids with side chain protecting groups were obtained in the course of solid-phase Edman degradation of a series of model peptidyl-resins in which the sequence framework Ala-X-Leu-Y-Ala- was used; X and Y correspond to two different protected amino acids. The peptides used are listed in Table III. Pth amino acids were identified by LC on a Microbondapak C_{18} column using gradient elution with acetonitrile in sodium acetate buffer, with detection at 254 nm. In the last two columns are tabulated the elution times for a mixture of standard Pth amino acids. [b] Areas are given relative to an equimolar amount of Pth-Nle (=1.0). The total amount of Pth amino acid expected in the Edman degradation cycles corresponding to positions X and Y in the sequence framework Ala-X-Leu-Y-Ala was calculated on the basis of the repetitive yield of Pth-Ala (cycles 1 and 5). Those areas underlined represent the major peak(s) encountered for the side chain protected Pth amino acids in the model peptidyl-resins as well as for a series of 11 peptidyl-resins varying in length from 8–117 residues. [c] These retention times are identical with those for the free Pth amino acid standards (see last two columns) suggesting that these peaks are the result of removal of the side chain protecting group during sequencing and/or synthesis. [d] The area in parentheses is for a peak detected at 313 nm, corresponding to (deprotected) Pth-dehydrothreonine (Margolies & Brauer, 1978).

where $j > i$. By use of this value of RY, the theoretical nanomolar values for the X and Y positions were calculated by extrapolation from the values of Pth-Ala at position 1. When the areas measured for the X and Y peaks are normalized to the theoretical values for the X and Y positions, it is possible to calculate peak areas for Pth-X and Pth-Y relative to the standard Pth-Nle. These are tabulated in Table IV where an equimolar amount of Pth-Nle has a peak area of 1.0. The peaks characteristic of the Pth derivatives of amino acids with side chain protecting groups found for these model peptides were also detected for the longer peptide(s) described below. However, the distribution of a given amino acid among several peaks varied, particularly in the case of Pth-*O*-benzylserine (see Table IV). It is possible that one of the multiple peaks encountered with each of several Pth amino

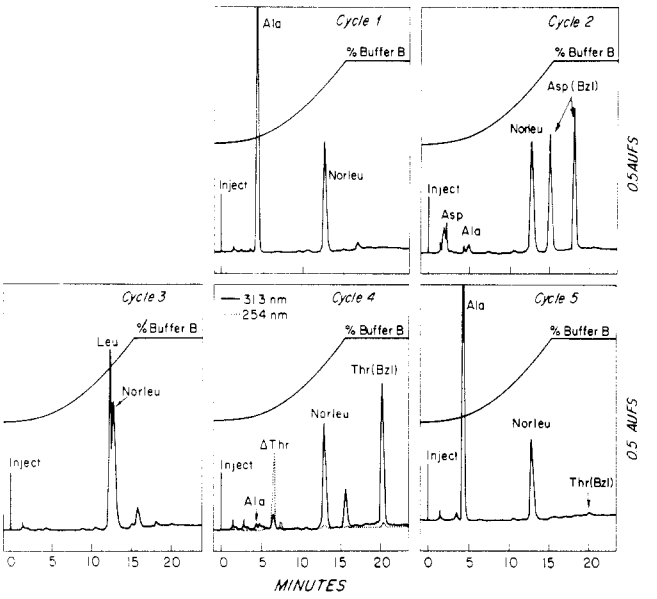

FIGURE 3: Characterization of Pth-Asp(OBzl) and Pth-Thr(OBzl) by high-pressure liquid chromatography after automatic solid-phase Edman degradation of Ala-Asp(OBzl)-Leu-Thr(OBzl)-Ala-Gly-$NHCH_2$-resin. In cycle 2, two peaks are characteristically observed when aspartyl residues are sequenced. A small amount of Pth-Asp is also obtained. In cycle 4, for threonine, in addition to two peaks which are detected at 254 nm, Pth-dehydrothreonine is also detected at 313 nm.

acids represents the unconverted thiazolinone, as in the case of proline described above where only partial conversion of Atz-Pro was encountered (see Figure 2). In the case of the side chain protected Pth derivatives of alanine, glutamic acid, threonine, and tryptophan, peaks with elution times characteristic of the deprotected Pth amino acids were also detected (see Table IV).

An example of chromatograms of Pth amino acids obtained from solid-phase Edman degradation of the peptidyl-resin Ala-Asp(OBzl)-Leu-Thr(OBzl)-Ala-Gly- is shown in Figure 3. In cycle 2, following conversion, a small amount of deprotected Pth-Asp is found as well as two peaks characteristic of derivatives of Asp(OBzl). In cycle 4, two peaks characteristic of the Pth derivatives of *O*-benzylthreonine are seen, as well as deprotected Pth-dehydrothreonine, the identification of which is enhanced by detection at 313 nm (Margolies & Brauer, 1978).

Solid-Phase Sequence Analysis of Peptidyl-resin Intermediates from Solid-Phase Syntheses of Antibody Fragments. Quantitative solid-phase Edman degradation was used to evaluate peptidyl-resin intermediates during the extended solid-phase synthesis of a 117-residue peptide. The amino acid sequence of this peptide corresponded to the heavy-chain variable region (V_H) of a homogeneous rabbit anti-pneumococcal antibody 3374 (Figure 1). At various points during the solid-phase synthesis (see Table V), samples of peptidyl-resin were taken from the reaction vessel for solid-phase Edman degradation. These sequencing experiments were designed to evaluate the most recently synthesized section and to overlap with the previously sequenced section. Thus it was possible to monitor retrospectively the progress of the solid-phase synthesis. When gross synthetic errors were detected, the synthesis was terminated. A new synthesis was restarted with certain modifications to overcome the errors which were detected.

In the first synthesis (peptidyl-resin A) a gross error was detected in the region of Thr^{101} to Trp^{107}. Examination of the various Pth amino acids quantified by LC as shown in Figure

Table V: Summary of Solid-Phase Edman Degradation of Peptidyl-resins Sampled[a] during Solid-Phase Synthesis of an Antibody Heavy-Chain Variable Region (Residues 2–118)

peptidyl resin sample	no. of aminoacyl residues	residues sequenced[b] (inclusive)	repetitive[c] yield (%)	preview observed: at residue no.	preview observed: at Edman cycle no.	preview observed: cumulative[d] preview (%)	av preview/cycle (%)
1	8	$Thr(OBzl)^{111} \longrightarrow Arg(Tos)^{118}$	99	Leu^{117}	6	4.9	0.82
2	18	$Thr(OBzl)^{101} \longrightarrow Leu^{112}$	96	Leu^{112}	11	2.3	0.21
3[e]	29	$Tyr(OCl_2Bzl)^{90} \longrightarrow Leu^{104}$	86	Leu^{104}	14	7.5	0.54
4	29	$Tyr(OCl_2Bzl)^{90} \longrightarrow Leu^{104}$	87	Leu^{104}	14	6.1	0.44
5	43	$Asp(OBzl)^{76} \longrightarrow Cys(Acm)^{92}$	93	Phe^{91}	15	1.5	0.10
6	56	$Lys(2\text{-}ClZ)^{63} \longrightarrow Leu^{77}$	91	Val^{75}	12	1.5	0.13
7	67	$Asn^{52} \longrightarrow Ile^{68}$	91	Phe^{66}	14	5.1	0.36
8	79	$Pro^{40} \longrightarrow Thr(OBzl)^{55}$	91	Val^{53}	13	4.6	0.35
9	89	$Ser(OBzl)^{30} \longrightarrow Gly^{43}$	93	Ala^{39}	9	4.9	0.54
10	99	$Thr(OBzl)^{20} \longrightarrow Ser(OBzl)^{34}$	95	Ala^{30}	12	3.9	0.33
11	109	$Leu^{10} \longrightarrow Val^{23}$	98	Val^{23}	13	4.0	0.31
12	117	$Ser^{2} \longrightarrow Thr(OBzl)^{12}$	91	Val^{11}	9	1.4	0.16

[a] Aliquots of peptidyl-resin were removed at intervals of 8–14 synthetic cycles and subjected to solid-phase Edman degradation. [b] The number of cycles of Edman degradation for each length peptide was chosen so as to overlap the sequence data for the next shortest peptide. [c] The repetitive yield for each sequencing experiment was based, when possible, on yields of a single stable Pth amino acid at two different cycles. In certain stretches of sequence (peptides of lengths 99, 79, 67, 56, and 43 residues). The repetitive yields are only approximations. since they are based on comparing two different stable Pth amino acids (see Figure 2). [d] Observed cumulative preview is given in percent for selected stable Pth amino acids located at or near the C terminus of the sequenced segment. [e] Edman degradation was repeated for this peptidyl-resin because of the relatively low repetitive yield.

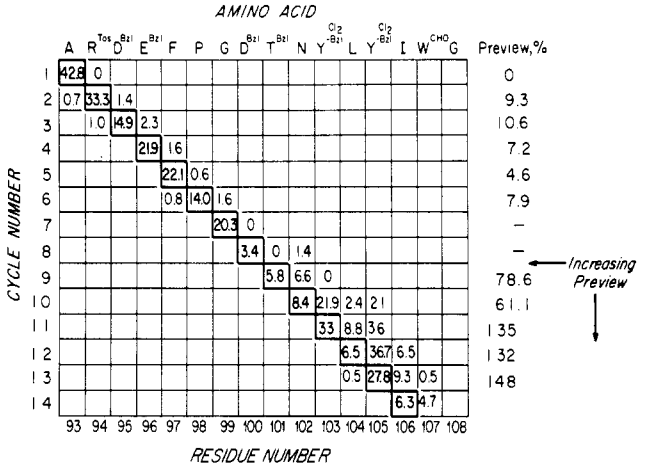

FIGURE 4: Quantitative solid-phase Edman degradation of synthetic peptidyl-resin A (26 residues) including residues 93–118 of the variable region of antibody 3374 heavy chain (see Figure 1). The target sequence is shown at the top in the one-letter code and the yields at each cycle of Edman degradation are listed in the ordinate. The expected correct sequence occupies a diagonal (boxed figures). Yields of Pth amino acids above the diagonal represent previews while yields below the diagonal are due to overlap. The percent preview for each cycle is listed at the right. The yield values given for side chain protected Pth amino acids are given in arbitrary units (area). The residue numbers in the target sequence are shown at the bottom.

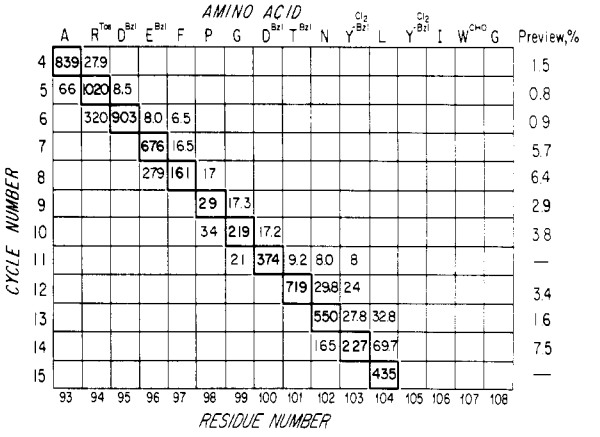

FIGURE 5: Quantitative solid-phase Edman degradation of synthetic peptidyl-resin C (29 residues) including residues 90–118 of the variable region of antibody 3374 heavy chain (see Figure 1). Data are shown beginning with cycle 4 (position 93) and may be compared to the results for the peptidyl-resin A (Figure 4) encompassing the same sequence region. See the legend to Figure 4 for further details.

4 indicated that beginning with the Edman degradation at cycle 9, a large increase in preview (79%) of Pth-Asn^{102} was observed along with the expected Pth-$Thr(OBzl)^{101}$, indicating that the attachment of Boc-$Thr(OBzl)^{101}$ was only 21% complete. Because the ninhydrin test was not performed during automated synthesis, this result could have arisen either from incomplete deprotection of the Boc group of Asn^{102} or from the incomplete DCC-mediated coupling of Boc-$Thr(OBzl)^{101}$ to Asn^{102}, or both. Further inspection of Figure 4 indicates that following cycle 9 the levels of premature detection of Pth amino acids (preview) continued to increase. At degradation cycle 13, the yield of Pth-Ile was larger than the yield of that residue at cycle 14 (position 106) where isoleucine is expected to be located. These data are consistent with a series of synthetic errors rather than an isolated failure to incorporate a single residue. The detection of these errors led to termination of the synthesis. The failure to completely incorporate $Thr(OBzl)^{101}$ proved to be due to incomplete coupling, based upon ninhydrin tests done during a repeat synthesis (peptidyl-resin B). This error was minimized in the synthesis of both peptidyl-resins B and C by repeating the coupling step until the ninhydrin test yielded a negative result. In Figure 5 is shown solid-phase Edman degradation data for peptidyl-resin C. Sequence analysis of this peptidyl-resin began at residue position 90 and thus overlapped the sequence shown for peptidyl-resin A (Figure 4). With the modifications in synthesis described above, the increase in preview between Thr^{101} and Trp^{107} previously observed is greatly reduced in the synthesis of peptidyl-resin C.

In the synthesis of peptidyl-resin B (43 residues) encompassing residues 76–118 of the 3374 heavy-chain sequence, an error involving an isolated total failure at one synthesis cycle occurred (see Figure 6). At cycle 5 of the degradation, the expected Pth-$Thr(OBzl)^{80}$ was not observed. The remainder of the sequence analysis, beginning at $Asp(OBzl)^{76}$, was characterized by relatively low levels of preview. This error of deletion was probably caused by an instrument malfunction.

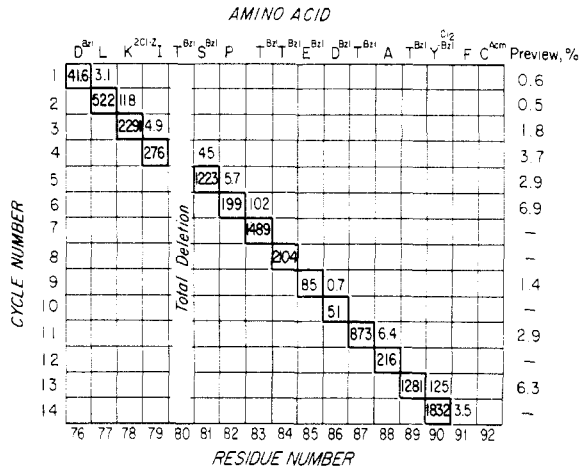

FIGURE 6: Quantitative solid-phase Edman degradation of synthetic peptidyl-resin B (43 residues) including residues 76–118 of the variable region of antibody 3374 heavy chain (see Figure 1). At cycle 5 the expected Pth-Thr(OBzl)^{80} was absent; instead Pth-Ser(OBzl)^{81} was identified indicating a total deletion of Thr(OBzl)^{80} during synthesis. This was thought due to omission of the program used for deprotection of Boc-Ser(OBzl)^{81}. See the legend to Figure 4 for further details.

We surmise that the program used for deprotection of the Boc-Ser(OBzl)^{81} was omitted. This example clearly establishes the value of solid-phase sequence analysis of peptidyl-resin samples. This is especially so in the case of an extended solid-phase synthesis where amino acid analysis could not be relied upon to provide evidence for a successful incorporation. Other amino acids in the same category as Thr(OBzl) are Ser(OBzl), Cys(Acm), and Trp(CHO). These residues are also partially or completely destroyed during acid hydrolysis of peptidyl-resin samples. Moreover, sequence analysis permits one to confirm the incorporation of an amino acid amide (asparaginyl or glutamyl) as distinct from the free acid form of these residues (aspartyl and glutamyl).

In contrast to peptidyl-resins A and B, the solid-phase assembly of peptidyl-resin C was judged successful by quantitative solid-phase Edman degradation. This judgment was made after sequencing the individual samples of peptidyl-resin intermediates which were taken at 10–15-residue intervals throughout the solid-phase synthesis. In Table V, the repetitive yield values for each solid-phase degradation experiment as well as the cumulative preview observed during each sequencing run are listed. Since the number of degradative cycles in each experiment was chosen to overlap with a previously sequenced region, we are confident that at each of the 117 positions in peptidyl-resin C only a single major Pth amino acid was found. Other Pth amino acids which were observed could be attributed to either sequence overlaps or to sequence previews. As indicated in Table V, significant levels of preview were observed consistently. This level of error caused by deletions amounted to an average of 0.4% per cycle. Thus, according to these calculations for a 116-cycle synthesis, $100 - (0.996^{116} \times 100)$ or 37% of the peptides which survived truncation contained one or more deletion errors.

On the basis of our experience with quantitative solid-phase Edman degradation, we have found (1) Kaiser ninhydrin monitoring of coupling will eliminate gross errors of deletion (≥10% preview) (compare peptidyl-resin A vs. peptidyl-resin C) and (2) despite repeated recoupling and acetylation to truncate uncoupled chains (see procedure for peptidyl-resins B and C), the low levels of preview could not be eliminated completely.

The last sequence experiment of peptidyl-resin C (Table V) provided additional evidence that solid-phase Edman degradation was performed efficiently even after 117 residues had been attached to the resin. The penultimate Leu residue at position 3 (see Figure 1) was tritium labeled. This provided the opportunity to determine the efficiency with which the solid-phase Edman degradation could be performed on a long peptide which was attached to resin. Following the final sequence analysis on the completed peptidyl-resin C, the resin was collected from the sequencer reaction column and hydrolyzed with propionic acid–HCl. An aliquot of the hydrolysate was counted to determine the level of the residual ^{3}H radioactivity. Similarly, the radioactivity in the hydrolysate of an unsequenced sample of peptidyl-resin C was determined. It was possible to normalize the two levels of radioactivity by taking advantage of the internal reference amino acid, aminobutyric acid. Previous work established that the quantity of Abu in a hydrolysate is proportional to the quantity of resin in the hydrolysis sample (Matsueda & Haber, 1980). Since peptidyl-resin C contained Abu as an internal reference amino acid, the ratio of ^{3}H cpm/Abu for sequenced and unsequenced peptidyl-resin C could be calculated. These were 104 before sequencing and 8.2 after sequencing. This indicated that 92% of the [^{3}H]Leu was removed from the resin in that solid-phase degradation experiment. This result demonstrated that even for a 117-residue peptide, solid-phase Edman degradation may be performed efficiently.

Discussion

The aim of the experiments reported here was to examine the utility of solid-phase Edman degradation for the characterization and thereby improvement of extended solid-phase peptide synthesis. For the synthesis of a short peptide it is expedient to cleave the peptide from the resin before evaluating the solid-phase assembly of the peptide. Thus, characterization and purification are performed together. However, when solid-phase synthesis of a longer peptide is attempted, it is preferable to characterize the assembly of the peptide while it is still attached to the solid support. Such characterization would be used to determine whether the synthesis should be continued or terminated.

The present work is distinguishable from the previous reports by Birr & Frank (1975) and Burton et al. (1979) in two ways. First, we demonstrate that solid-phase Edman degradation was performed efficiently on available peptide chains. Sequence analysis of only a fraction of the synthetic peptides on the solid support would have limited value. By using tritium-labeled peptidyl-resins, we have shown that the efficiency of solid-phase Edman degradation was >90% for a model peptidyl-resin as well as for a 117-residue peptidyl-resin. Second, we recognized that acid instability of the peptide to resin linkage contributes to the lowering of the sequencing repetitive yields when the –oxymethyl– linkage is used. When the –oxymethyl-Pam– linkage is used, the sequencing repetitive yields averaged 95% for model peptides (Matsueda & Margolies, 1979). Thus, we recommend the use of more acid-resistant peptide to resin linkages for purposes of solid-phase sequencing as well as for increased synthetic yields (Sparrow, 1976). The use of the benzhydrylamine resin also provides improved repetitive yields comparable to the Pam-resins (G. R. Matsueda, unpublished observations).

In order to evaluate the synthetic peptidyl-resin directly, it was necessary to prepare and characterize 10 nonnatural Pth amino acids which occurred in various V_H-3374 peptidyl-resin intermediates. Rather than using classical solution methods of synthesis, we prepared the various reference Pth amino acids as components of five carrier peptidyl-resins produced in the

same fashion as the target V_H-3374 peptidyl-resin. This approach was used to minimize the changes in LC patterns of unstable Pth amino acids and those which gave multiple peaks. Some derivatives such as Pth-Arg(Tos) or Pth-Cys(Acm) were detected by LC as single major peaks and were useful, therefore, for quantification as well as identification. On the other hand, derivatives such as Pth-Ser(OBzl) or Pth-Thr(OBzl) yielded two to four peaks by LC and thus were useful for identification but not absolute quantification. Nonetheless, a cycle to cycle comparison of these amino acids was possible due to reproducibility of the LC patterns of the reference Pth derivatives which yielded multiple peaks within any sequencing experiment.

During repetitive Edman degradation, a gradual increase in "background" amino acids is seen owing, probably, to acidolysis of peptide bonds occurring during cleavage (Edman & Begg, 1967). The total amount of background seen is proportional to the length of the peptide being sequenced and the number of cycles of acid exposure. Possible increase in background in the larger peptides studied (see Table V) is lessened by the fact that each sequencing experiment was limited to 8–14 cycles. We defined preview as occurring when the amount of a Pth amino acid identified in a cycle earlier than anticipated was significantly higher than the increase in background expected. When the synthetic deletion errors are small, preview cannot be distinguished from background. The figures given for preview in Figures 4–6 are indicated only for single-deletion errors. Multiple-deletion errors were detected (see cycle 8, Figure 4), but the statistical assignment of error subsequences containing more than one deletion is complex.

The utility of comparing synthetic peptides at each cycle n for sequence uniformity on the basis of criteria of finding the nth Pth amino acid at cycle $n - 1$ is demonstrated by comparing the analyses of the two peptides presented in Figures 4 and 5. Here, modification of the synthetic protocol used resulted in unequivocal improvement in the homogeneity of the peptidyl-resin C (see Figure 5). Calculations of preview are thus more useful for *comparing* synthetic products for sequence uniformity than for attempting to state a precise degree of sequence homogeneity for any single peptide.

While sequence analysis of a peptidyl-resin evaluates the sequence uniformity of the synthetic product, it cannot give any information about the extent of amino acid incorporation. For this, amino acid analysis of peptidyl-resin samples is required. Several hydrolytic procedures have been employed successfully (Stewart & Young, 1969; Gutte & Merrifield, 1971; Scotchler et al., 1970). These procedures in general suffer because they cannot confirm the incorporation of an amino acid which is either destroyed during the acid hydrolysis step or expected to give a small increment upon what has already been incorporated. Fortunately, in each of these situations LC of the corresponding Pth derivative obtained from a sequencing experiment confirms the identity of the particular residue incorporated.

It is likely that solid-phase Edman degradation will be useful in conjunction with those synthetic strategies which were proposed for the selective "rescue" of completed peptide chains. By chemically modifying the amino groups which survived truncation, it was possible to purify selectively those chains in the presence of shorter, truncated chains (Krieger et al., 1976; Merrifield & Bach, 1978). Included among the rescued completed chains, however, are peptide chains which may contain errors of deletion. Therefore, these strategies place a premium on sequence homogeneity. Thus, solid-phase Edman degradation is useful for the selection of synthetic protocols which will minimize synthetic errors due to the deletion of amino acid residues.

Acknowledgments

For skilled technical assistance, we thank Stephan A. Gaehde and David M. Steiman for the synthesis of peptides, Leslie Dotson, Christine Oman, and Andrew Brauer for solid-phase sequence analysis, and John Wechsler for amino acid analysis. G.R.M. gratefully acknowledges Dr. Alexander R. Mitchell for advice regarding preparation and use of the Pam handle and the aminomethyl-resin.

References

Birr, C., & Frank, R. (1975) *FEBS Lett. 55*, 68.

Burton, J., Topper, R., & Ehrlich, P. H. (1979) in *Peptides* (Gross, E., & Meienhofer, J., Eds.) p 605, Pierce Chemical Co., Rockford, IL.

Chang, J. K., Shimizu, M., & Wang, S. S. (1976) *J. Org. Chem. 41*, 3255.

Chapman, T. M., & Kleid, D. G. (1973) *J. Chem. Soc., Chem. Commun.*, 193.

Coy, D. H., Gill, P., Kastin, A. J., Dupont, A., Cusan, L., Labrie, F., Britton, D., & Fertel, R. (1977) in *Peptides* (Goodman, M., & Meienhofer, J., Eds.) p 107, Wiley, New York.

de Vries, J. X., Frank, R., & Birr, C. (1975) *FEBS Lett. 55*, 65.

Edman, P. (1957) *Acta Chem. Scand. 4*, 283.

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

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.) 2*, 424.

Fankhauser, P., Fries, P., Stahala, P., & Brenner, M. (1974) *Helv. Chim. Acta 57*, 271.

Frechet, J. M., & Scheurch, C. (1971) *J. Am. Chem. Soc. 93*, 492.

Gutte, E., & Merrifield, R. B. (1971) *J. Biol. Chem. 246*, 1922.

Hagenmaier, H., & Frank, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1973.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595.

Kreiger, D. E., Erickson, B. W., & Merrifield, R. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3160.

Laursen, R. A. (1971) *Eur. J. Biochem. 20*, 89.

Laursen, R. A., Bonner, A. G., & Horn, M. J. (1975) *Instrumentation in Amino Acid Sequence Analysis*, p 73, Academic Press, London.

Margolies, M. N., & Brauer, A. W. (1978) *J. Chromatogr. 148*, 429.

Margolies, M. N., & Haber, E. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1852.

Matsueda, G. R., & Margolies, M. N. (1979) *FEBS Lett. 106*, 89.

Matsueda, G. R., & Haber, E. (1980) *Anal. Biochem. 104*, 215.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149.

Merrifield, R. B., & Bach, A. E. (1978) *J. Org. Chem. 43*, 4808.

Mitchell, A., Erickson, B., Ryabtsev, M., Hodges, R., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357.

Moroder, L., Hallet, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651.

Niall, H. D., Tregear, G. W., & Jacobs, J. (1972) *Chem. Biol. Pept., Proc. Am. Pept. Symp., 3rd*, 695.

Scotchler, J., Lozier, R., & Robinson, A. B. (1970) *J. Org. Chem. 35*, 3151.

Sparrow, J. P. (1976) *J. Org. Chem. 41*, 1350.
Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, p 53, W. H. Freeman, San Francisco, CA.
Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., D'Riordan, J. H., & Potts, J. T., Jr. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 415.
van Rietschoten, J., Granier, C., Rochat, H., Lissitzky, S., & Miranda, F. (1975) *Eur. J. Biochem. 56*, 35.
Westall, F. C., Scotchler, J., & Robinson, A. B. (1972) *J. Org. Chem. 37*, 3363.
Yamashiro, D., & Li, C. H. (1973) *J. Am. Chem. Soc. 95*, 1310.
Yamashiro, D., & Li, C. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4549.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569.

Light-Scattering Investigation of the Subunit Dissociation of *Homarus americanus* Hemocyanin. Effects of Salts and Ureas†

Theodore T. Herskovits,* Richard C. San George, and Lawson J. Erhunmwunsee

ABSTRACT: The subunit dissociation of the hemocyanin of the lobster, *Homarus americanus*, by the various salts of the Hofmeister series and the hydrophobic reagents of the urea–guanidinium chloride (GdmCl) class was investigated by laser light scattering molecular weight measurements. The dissociations of the hemocyanin dodecamers to hexamers by the various salts and the lower members of the urea series are found to be rapid and reversible, as predicted by the mass action law for monomer–dimer type of reactions. The salts are found to be very effective dissociating agents with the usual order of increasing effectiveness, $Cl^- < Br^- < I^- < ClO_4^-$, SCN^-. The ureas and GdmCl are found to be relatively ineffective dissociating reagents. In addition, the ureas show a decreasing order of effectiveness in going from urea to methyl-, ethyl-, and propylurea. This suggests that hydrophobic interactions are not the dominant stabilizing forces between the pairs of hexamers that form the dodecameric structure. Polar and ionic interactions appear to be the major stabilizing forces of the dodecameric structure. The use of equations derived for predicting the effects of dissociating reagents and salts on the structure of subunit proteins [Herskovits, T. T., & Ibanez, V. S. (1976) *Biochemistry 15*, 5715–5721] together with binding and Setschenow constants based on model amino acid data is found to give good account of the dissociation behavior observed with the salts, urea, and methylurea in the presence of calcium ion at both pH 7.8 and pH 9.5. The apparent number of amino acids at the contact areas of the hexamers, N_{app}, required to fit the dissociation data were found to be 24 ± 8 at pH 7.8 and 23 ± 4 at pH 9.5. However, because of the possible effects of molecular microheterogeneity, the estimates of amino acids at the contact areas must be viewed with caution, depending on further investigations.

The hemocyanins of the arthropod species are multisubunit proteins of varying complexity, assembled from one to eight basic hexameric units (Bijholt et al., 1979; Klarman et al., 1979; Jeffrey, 1979). Thus, the hemocyanins isolated from the hemolymph of the lobsters *Panulirus interruptus* and *Panulirus vulgaris* consist essentially of the single hexameric species (Kuiper et al., 1975; Van Bruggen et al., 1963) whereas the hemocyanin of the horseshoe crab, *Limulus polyphemus*, is an assemblage of six hexameric units (Bijholt et al., 1979). The hemocyanin of the lobster *Homarus americanus* has an intermediate structure, consisting of two basic hexameric units (Morimoto & Kegeles, 1971). With the hemocyanin of the ghost shrimp, *Callinassa californiensis* (Roxby et al., 1974), the lobster hemocyanin represents one of the few hemocyanins that exhibit ligand-mediated monomer-dimer type of association-dissociation equilibria.

Changes in solvent conditions, such as pH, the concentration of divalent ions, and the concentration of dissociating reagents, are known to alter the state of association of the different hemocyanin species. For the past several years, we have investigated the dissociation behavior of subunit proteins by using hydrophobic reagents and salts as probes of the contact areas of the subunits (Elbaum & Herskovits, 1974; Herskovits et al., 1977, 1980). In the case of the hemocyanin of the crab, *Callinectes sapidus*, light scattering molecular weight measurements have shown that the ureas and the Hofmeister series of salts dissociate both the hexameric and the dodecameric components of this hemocyanin (Herskovits et al., 1981). The salts were found to follow the usual order of effectiveness as dissociating agents of the dodecameric component whereas the ureas showed an inverse order of decreasing effectiveness with increasing hydrocarbon content of the urea, suggesting that polar and ionic interactions are relatively more important than hydrophobic interactions for the stabilization of the dodecameric structure. Unfortunately, the hemocyanin components of *Callinectes* were not found to exhibit true equilibrium behavior of the dissociation species, as suggested by the lack of concentration dependence of the light-scattering molecular weights upon dilution of the protein components. Such effects have been noted with most of the hemocyanins and have been attributed to the effects of microheterogeneity of the dissociating species (Di Giamberardino, 1967; Konings et al., 1969; Siezen & Van Driel, 1973). These findings did not allow us to analyze the dissociation data in any significant detail. The fact that the *Homarus* hemocyanin exhibits rapid and reversible dodecamer–hexamer equilibrium (Morimoto & Kegeles, 1971) and the desire to investigate the dissociation behavior of other hemocyanins regarding the question of the forces that hold the subunits together have prompted the

† From the Department of Chemistry, Fordham University, Bronx, New York 10458. *Received August 19, 1980.* This work was supported by Grant AM 20156 from the National Institutes of Health, U.S. Public Health Service.

0006-2960/81/0420-2580$01.25/0