

# Separation of Large Denatured Peptides by Reverse Phase High Performance Liquid Chromatography

TRIFLUOROACETIC ACID AS A PEPTIDE SOLVENT\*

(Received for publication, August 4, 1980)

Walter C. Mahoney and Mark A. Hermodson

From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

A method employing reverse phase high performance liquid chromatography has been developed for the purification of large denatured peptides. The cyanogen bromide fragments of hemoglobin  $\alpha$ ,  $\beta$ , and  $\gamma$  chains (13 to 91 residues in length) could be separated and recovered in 71 to 95% yields using LiChrosorb C-8 columns and a binary solvent system consisting of 0.013 M trifluoroacetic acid in water and a limiting organic solvent. This procedure permits the nondestructive detection of peptides at low wavelength (210 nm) on as little as 50 ng of sample. The peptides are recovered directly by evaporation or lyophilization. An elutropic solvent series for the separation of large and small peptides on reverse phase supports is also described.

The isolation of pure peptides from mixtures generated by enzymatic or chemical digestion of proteins has always been the most costly procedure both in time and in expenditure of materials in the determination of an amino acid sequence. The introduction of the sequenator (1) led to a shift in emphasis from generating and purifying a large number of small peptides suitable for manual sequencing to generating and purifying a small number of large fragments (30 to 100 residues in length) suitable for automated sequencing. While the former technique was complicated and time-consuming due to the complexity of the mixture, the latter proved difficult due to the highly hydrophobic nature of the large fragments, which made them difficult to dissolve and manipulate in aqueous solutions.

The most successful method for the fractionation of large denatured peptides employs gel filtration in the presence of high concentrations of organic acids (e.g. 1 to 4 M formic or acetic acid) or denaturants (e.g. urea, guanidine·HCl, sodium dodecyl sulfate, or chaotropic salts). Unfortunately, most of these solutions are not transparent at the wavelengths most appropriate for the detection of peptides (210 to 230 nm). While a crude chromatographic profile may be obtained by following the 254 nm and 280 nm absorbances, these depend on the presence of aromatic residues which are not uniformly distributed in the peptides. To ensure detection of all the peptides, post-column base hydrolysis and reaction of the amino acids with fluorescamine, *o*-phthalaldehyde, or ninhydrin is commonly performed on small aliquots of each fraction (2-5). Gel filtration or cation exchange columns run in weak phosphoric acid solutions may be successfully employed for

some separations (6), but while this permits UV detection at 206 nm and higher, large peptides are frequently insoluble in phosphoric acid, and the solvent is not totally volatile and requires a cumbersome desalting procedure. Gel filtration at high pH (9 to 11) may be accomplished using buffers which are volatile and which have a low UV cutoff (7), but peptide insolubility and poor chromatographic characteristics limit the utility of this procedure.

The use of HPLC<sup>1</sup> to isolate peptides has gained increasing attention from protein chemists recently. Unfortunately, while there has been considerable success in purifying native proteins (8, 9) and small peptides obtained by extensive enzymatic proteolysis (10-14), the results have not been as encouraging with large peptides.

Recently, Gerber *et al.* (15) and Takagaki *et al.* (16) have reported the successful isolation of large peptides for the sequence analysis of bacteriorhodopsin and rabbit liver cytochrome *b<sub>5</sub>*, respectively. They used reverse phase HPLC on C-18 columns with a mobile phase consisting of formic acid, water, and ethanol. Here, too, peptide detection was only possible at insensitive UV wavelengths (280 nm) due to the presence of formic acid.

We report here a general method for the purification of large peptides by reverse phase HPLC where the solvent system meets four exacting criteria: 1) it is an excellent solvent for large peptides; 2) it is volatile for easy sample recovery; 3) it is transparent enough to allow very sensitive, nondestructive detection of any peptide at low wavelength (210 to 230 nm); and 4) it employs several possible solvents for maximum resolution of the peptide mixture of interest. This system will make the resolution and nondestructive detection of the peptides in a submilligram sample possible and will greatly facilitate sequence analysis of proteins available in only small amounts.

## EXPERIMENTAL PROCEDURES

**Chemicals**—Glass-distilled methanol, acetonitrile, dioxane, tetrahydrofuran, 2-propanol, and 1-propanol were purchased from Burdick and Jackson. Absolute ethanol was obtained from U.S. Industrial Chemical Co. Sequenal grade trifluoroacetic acid was purchased from Pierce. Cyanogen bromide was obtained from Eastman. All other chemicals were of the highest purity commercially available.

**Hemoglobin Preparations**—Whole human globins from the hemolysates of three fetuses and one neonate were deheated by acid-acetone precipitation (17) and used without further purification. Globin  $\alpha$ ,  $\beta$ , and  $\gamma$  chains were isolated as previously described (18-20). Following reduction and S-alkylation (21), salt-free lyophilized chains and globins were dissolved in 70% formic acid and cleaved with cyanogen bromide (22). The peptides were recovered by lyophilization and dissolved in 0.1% (v/v, 0.013 M) trifluoroacetic acid in water. Protein concentration ranged from 1 to 2 mg/ml.

\* This work was supported by United States Public Health Service Grant GM24602. This is Journal paper 8151 from the Purdue University Agricultural Experimental Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> The abbreviation used is: HPLC, high performance liquid chromatography.

**Designation of Fragments**—The cyanogen bromide fragments were numbered consecutively from those containing the NH<sub>2</sub>-terminal sequence to those containing the COOH-terminal sequence of the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. Thus, the  $\alpha$  chain fragments are identified as  $\alpha$ CB-1 (residues 1 to 32),  $\alpha$ CB-2 (residues 33 to 76), and  $\alpha$ CB-3 (residues 77 to 141). The  $\beta$ -chain fragments are  $\beta$ CB-1 (residues 1 to 55) and  $\beta$ CB-2 (residues 56 to 146), and the  $\gamma$  chain fragments are  $\gamma$ CB-1 (residues 1 to 55),  $\gamma$ CB-2 (residues 56 to 133), and  $\gamma$ CB-3 (residues 134 to 146).

**Amino Acid Analysis**—Peptides were hydrolyzed in glass-distilled 6 N HCl *in vacuo* at 110°C for 24 h. All analyses were performed on a Durrum D-500 amino acid analyzer according to standard procedures.

**High Performance Liquid Chromatography**—Separation of the cyanogen bromide fragments was carried out by reverse phase HPLC on a Varian model 5000 HPLC equipped with a Rheodyne model 7125 sample injector with a 100- $\mu$ l sample loop, a Vari-Chrom detector, and a Linear model 260/MM chart recorder. A slight amount of back pressure was introduced in the flow cell by the application of a pinchcock clamp to the eluate line. This prevented the formation of bubbles due to solvent out-gassing. The analyses were performed on a LiChrosorb RP-8 (4.6  $\times$  250 mm), 5 $\mu$ m, column (Brownlee Labs).

**HPLC Column Maintenance**—The efficiency of any column will invariably deteriorate with use. The major symptoms that indicate that column repair is necessary are an increase in inlet pressure and peak broadening with a drop in resolution. These problems are usually caused by a clogged inlet frit, build up of insoluble material at the top of the column bed, or settling of the bed. The frit may be cleaned by removing and boiling it in concentrated HNO<sub>3</sub>/H<sub>2</sub>O (1:1). If discoloration is observed at the top of the bed, remove the discolored packing material and firmly replace it with moist stationary phase with a spatula. This will usually restore the column characteristics to those of a new column.

## RESULTS

**Peptide Solubility**—Low concentrations (0.01 M) of trifluoroacetic acid in water effectively dissolved all the peptides tested, even at the high concentrations of organic solvents necessary for elution of the peptides from reverse phase columns. Similar perfluorinated acids such as pentafluoropropionic were equally effective, but the price and ready availability of trifluoroacetic acid in highly purified form made it the better choice. Trifluoroacetic acid has two major advantages over previous peptide solvents (*e.g.* formic acid and phosphoric acid) used in HPLC. First, trifluoroacetic acid is volatile (b.p. 71–72°C), and second, it has a low UV cutoff. The practical UV limit (optical density, 0.3) of a 0.1% (0.013 M) solution of trifluoroacetic acid in water is 216 nm, while that for a 0.05% solution is 208 nm.

**Solvent Selection: an Elutropic Series**—The 13-residue COOH-terminal cyanogen bromide peptide of human  $\gamma$  chain ( $\gamma$ CB-3) was employed to test the relative effectiveness of various solvents for eluting peptides from the C-8 column.

TABLE I

Retention time of  $\gamma$ CB-3 as a function of solvent

Temperature was maintained at 30°C, flow rate was 0.7 ml/min, and the rate of solvent change was 1.6%/min. Pressure varied with solvent viscosity.

Solvent	Elution time min	Solvent %	Solvent UV cut- off <sup>a</sup>	Solvent viscosity <sup>b</sup> (30°C)
Methanol	34.6	57.6	205	0.54
Acetonitrile	28.1	46.8	190	0.33
Ethanol	27.6	46.0	205	0.99
Dioxane	26.2	43.6	215	1.09
Tetrahydrofuran	26.1	43.4	212	0.46
2-Propanol	22.5	37.5	205	1.77
1-Propanol	20.3	33.8	210	1.72

<sup>a</sup> Solvent UV cutoffs are from Burdick and Jackson Laboratories and Riddick and Bunger (26).

<sup>b</sup> Solvent viscosities expressed as dynamic viscosity are from Riddick and Bunger (26). Values are given in centipoise.

TABLE II

The influence of mobile phase construction on peptide retention times

Samples (100  $\mu$ g) were dissolved in 0.1% trifluoroacetic acid. Flow rate, 0.7 ml/min; temperature, 30°C; rate of solvent change, 1.6%/min

Limiting mobile phase <sup>a</sup>	Retention times <sup>b</sup>		
	$\alpha$ CB-1	$\alpha$ CB-2	$\alpha$ CB-3
			min
1-Propanol	18.3	28.0	34.0
2-Propanol	21.6	34.3	39.2
70% 1-propanol, 30% tetrahydrofuran	19.2	30.9	37.0
70% 1-propanol, 30% acetonitrile	26.6	36.8	44.0
50% 1-propanol, 50% tetrahydrofuran	22.3	33.0	40.0
Tetrahydrofuran	33.2	41.8	47.4
Dioxane	34.1	42.0	49.6
Ethanol	48.1	NE <sup>c</sup>	NE
Acetonitrile	49.0	NE	NE
Methanol	NE	NE	NE

<sup>a</sup> All solvents contained 0.1% trifluoroacetic acid to prevent base-line shift.

<sup>b</sup> The peptides produced by cyanogen bromide cleavage of hemoglobin  $\alpha$  chain were used throughout.

<sup>c</sup> NE, not eluted.

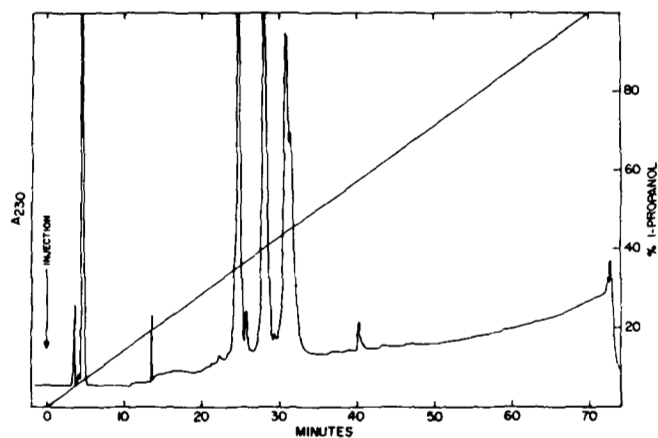


FIG. 1. Resolution of the cyanogen bromide peptides of the  $\alpha$  chain of human hemoglobin.  $\alpha$ CB-1,  $\alpha$ CB-2, and  $\alpha$ CB-3 elute at 25, 28, and 31 min, respectively. Sample, 200  $\mu$ g of the digest dissolved in 0.013 M trifluoroacetic acid (100  $\mu$ l); temperature, 28°C; flow, 0.7 ml/min. The starting solvent was 0.013 M trifluoroacetic acid in water and a gradient of 1-propanol containing 0.013 M trifluoroacetic acid was employed as indicated. The absorbance range was 2 A full scale.

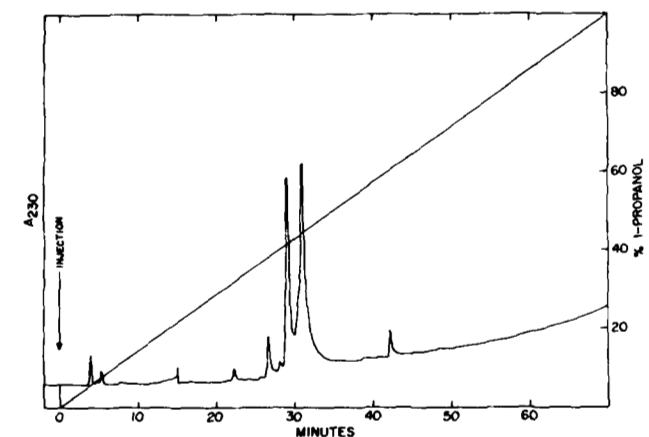


FIG. 2. Separation of the two cyanogen bromide peptides of human hemoglobin  $\beta$  chain under the conditions used in Fig. 1.  $\beta$ CB-2 elutes at 29.5 min and  $\beta$ CB-1 at 31 min.

Identical linear gradients of each were formed with 0.1% (v/v) trifluoroacetic acid in both the water and the organic phases. An elutropic solvent series was determined from the solvent concentrations of each required to elute the peptide from the column (Table I). For example, a methanol concentration of 57.6% was required to elute  $\gamma$ CB-3 compared to 33.8% for 1-propanol. Subsequently, it was found that the results obtained on  $\gamma$ CB-3 were applicable to peptides of every size examined (Table II). Since many peptides bind very strongly to reverse phase columns, it was not possible to elute the largest and most hydrophobic peptides using the weakest solvents (methanol, acetonitrile, etc.) regardless of the solvent concentration. Thus, gradients of 1-propanol or 2-propanol were necessary for elution of many peptide mixtures.

Representative chromatograms showing the separation of the cyanogen bromide peptides of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains are

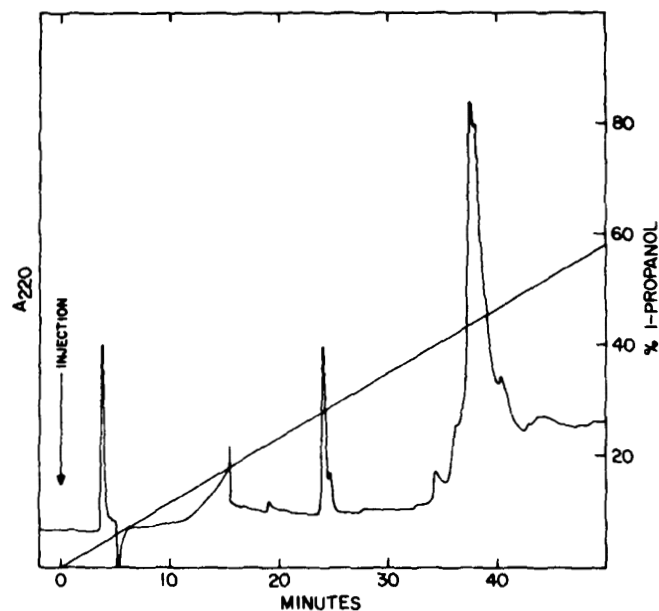


FIG. 3. Separation of the cyanogen bromide fragments of human hemoglobin  $\gamma$  chain under the conditions used in Fig. 1. Peptide  $\gamma$ CB-3 elutes at 24 min followed by  $\gamma$ CB-1 and  $\gamma$ CB-2 co-eluting at 39 min.

presented in Fig. 1, 2, and 3, respectively. Amino acid compositions and the yields of the peptides are presented in Table III. Throughout, the yields from the HPLC were more than 70% of the amount injected and frequently were over 90%, which compares favorably with gel filtration.

The retention times of the  $\alpha$  chain cyanogen bromide fragments relative to each other can be altered by the use of solvent mixtures (Table II) or by increasing or decreasing the rate of solvent change. Gradient programming, combined with the proper solvent choice, adds a wide range of flexibility to achieve peptide separations. The best resolution of peptides on reverse phase columns occurs in the solvent range of 15 to 60%. Thus, short peptides which elute below 15% propanol concentrations are best resolved in acetonitrile or ethanol gradients where the eluting solvent concentrations are increased. Conversely, peptides eluting in very high solvent concentrations frequently emerge in broad, poorly resolved

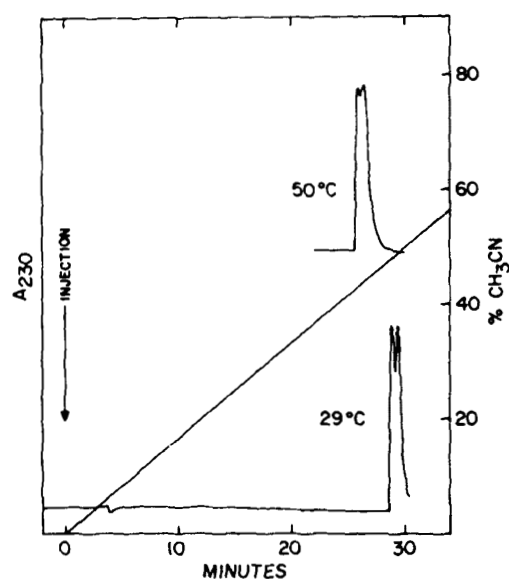


FIG. 4. Influence of temperature on the resolution of  $\gamma$ CB-3 (eluting first) and  $\alpha$ - $\gamma$ CB-3. Experimental procedure is as in Fig. 1, except acetonitrile was used to form the gradient.

TABLE III  
Amino acid compositions of peptides eluted from reverse phase HPLC

Fragment: Yield:	$\alpha$ -CB-1 93%	$\alpha$ -CB-2 82%	$\alpha$ -CB-3 75%	$\beta$ -CB-1 86%	$\beta$ -CB-2 77%	$\gamma$ CB-1&2 71%	$\gamma$ -CB-3 95%
Asp	2.1 (2) <sup>a</sup>	5.0 (5)	4.8 (5)	3.8 (4)	8.7 (9)	12.8 (13)	
Thr	0.9 (1)	3.5 (4)	3.6 (4)	3.6 (4)	2.6 (3)	8.2 (9)	0.9 (1)
Ser	1.6 (1)	2.5 (3)	6.2 (7)	2.1 (3)	1.5 (2)	7.0 (8)	2.5 (3)
Glu	3.0 (3)	1.2 (1)	0.8 (1)	5.5 (6)	4.8 (5)	11.9 (12)	
Pro	0.9 (1)	2.1 (2)	3.9 (4)	2.7 (3)	3.8 (4)	4.9 (4)	
Gly	4.3 (4)	3.3 (3)		4.8 (5)	7.6 (8)	11.9 (12)	0.8 (0.8)
Ala	6.7 (7)	3.8 (4)	8.8 (9)	3.8 (4)	10.8 (11)	7.6 (8)	2.2 (2.2)
Val	2.6 (3)	5.2 (5)	5.6 (6)	7.8 (8)	9.7 (10)	11.5 (11)	1.8 (2)
Met <sup>b</sup>	0.7 (1)	0.8 (1)		0.6 (1)		1.7 (2)	
Ile	0.5 (0)					3.8 (4)	
Leu	1.8 (2)	2.9 (3)	12.1 (13)	5.6 (6)	11.9 (12)	15.1 (16)	1.0 (1)
Tyr	0.8 (1)	0.9 (1)	0.9 (1)	0.8 (1)	1.9 (2)	0.8 (1)	
Phe		3.4 (4)	2.8 (3)	2.9 (3)	4.8 (5)	7.4 (8)	
His	0.6 (1)	3.6 (4)	4.9 (5)	0.8 (1)	8.0 (8)	5.9 (6)	0.9 (1)
Lys	2.8 (3)	3.8 (4)	3.8 (4)	1.6 (2)	8.7 (9)	11.8 (12)	
Arg	1.0 (1)		1.8 (2)	1.8 (2)	0.9 (1)	1.7 (2)	0.9 (1)
Cys <sup>c</sup>			0.8 (1)		1.6 (2)	1.1 (1)	
Trp	ND <sup>d</sup>	ND	ND	ND	ND	ND	ND

<sup>a</sup> Values in parentheses refer to compositions determined from the sequence (27).

<sup>b</sup> Detected as homoserine and homoserine lactone.

<sup>c</sup> Detected as *S*-(pyridylethyl)cysteine.

<sup>d</sup> ND, not determined.

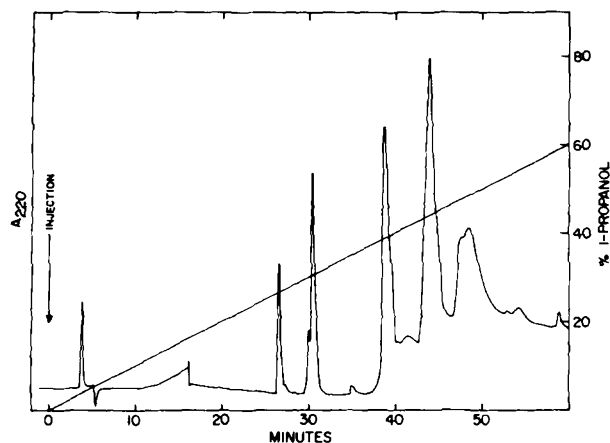


FIG. 5. Separation of the cyanogen bromide fragments of fetal globin isolated from human embryos 161 to 170 mm in length (18 to 19 weeks old). The fragments are in the order of elution:  $\gamma$ CB-3,  $\alpha$ CB-1,  $\gamma$ CB-1 and  $\gamma$ CB-2 (eluting together),  $\alpha$ CB-2, and  $\alpha$ CB-3 at 28, 31, 39, 45, and 49 min, respectively. Experimental procedure is as in Fig. 1.

TABLE IV

Comparison of  $\gamma$ CB-3 preparations from HPLC or gel filtration to determine  $^G\gamma$  chain percentage in Hb F

Purified  $\gamma$ CB-3 (by either method) was hydrolyzed and the Ala and Gly amounts were determined by amino acid analysis. Values were determined by gel filtration as previously described (28).

Hb F sample	HPLC		Gel filtered		$^G\gamma$ chains
	Gly	Ala	Gly	Ala	
	Residues/CB <sub>n</sub> peptides				%
R.B.	0.79	2.20	0.80	2.21	79/80
16	0.80	2.37	0.81	2.25	80/81
18	0.74	2.35	0.71	2.30	74/71
4	0.79	2.34	0.66	2.40	79/66

peaks and should be chromatographed in a more effective eluting solvent.

**Temperature**—The effect of increasing temperature was to decrease the retention times and the back pressure, increase the incidence of artifacts such as multiple peaks in pure samples, and decrease the resolution of the analysis. This was found for both small tryptic peptides and large cyanogen bromide peptides. Fig. 4 illustrates the separation of  $^A\gamma$ CB-3 (with Ala in position 136) and  $^G\gamma$ CB-3 (with Gly in position 136) at 30 and 50°C.

**Flow Rate**—The resolution of peptides on the columns used in this investigation was optimal at 0.7 to 0.8 ml/min. Even relatively viscous solvents such as 1-propanol did not produce excessive back pressures at such low flow rates. Faster flow rates could be used with acetonitrile without causing high back pressures, but this did not improve the resolution.

**Reproducibility**—Several fetal globins were chromatographed as in Fig. 5, and the  $\gamma$ CB-3 peak was collected. These were hydrolyzed and subjected to amino acid analyses in order to determine the ratio of  $^G\gamma$  to  $^A\gamma$  chains in the sample (Table IV). The data obtained from these analyses were entirely consistent with those from the same samples performed by isolation of the  $\gamma$ CB-3 peaks on Sephadex columns. The HPLC analyses took 26 min each, the Sephadex columns took 24 h or more.

#### DISCUSSION

The application of HPLC for peptide separations has several advantages over other methods of peptide purification, including easy sample recovery (by evaporation or lyophilization), flexibility, resolution, speed, sensitivity, high yields, and adaptability to quantitative analysis of peptide recovery.

While not all peptide mixtures can be resolved using reverse phase HPLC ( $\gamma$ CB-1 and  $\gamma$ CB-2 could not be separated, Fig. 2), we have found the procedure to be a useful and complementary addition to the limited set of methods currently available to purify large peptides.

The order of elution of peptides from reverse phase columns is somewhat dependent on the size of the peptide. This is probably due to the more hydrophobic character of large peptides relative to short peptides. An exception to this rule is the 91-residue fragment of  $\beta$  chain ( $\beta$ CB-2) which elutes before the 55-residue fragment ( $\beta$ CB-1) (Fig. 2). The larger fragment in this case is quite hydrophilic, while the shorter is very hydrophobic. The order in which hydrophilic and hydrophobic residues appear in a peptide has also been shown to influence the elution profile of small peptides (12). With large peptides, the contribution of amino acid sequence to retention time is difficult to evaluate. Assuming that large peptides still maintain some partly folded configuration, retention time could be influenced by the nature of the residues available for interaction with the stationary phase and thus could be dependent upon sequence-directed conformation, for example.

The manner in which trifluoroacetic acid interacts with peptides and maintains their solubility is not known. It is a powerful solvent as judged by the solubility of the peptides in mixtures containing up to 60% or more of organic solvents like acetonitrile where peptides are normally very insoluble. Ion pairing may play some role in this phenomenon, but trifluoroacetic acid has a larger effect than other ion-pairing agents.

The use of 1-propanol for the elution of peptides from reverse phase supports was introduced by Rubinstein *et al.* (23). Since then, gradients of 1-propanol have been utilized several times for protein (9) and peptide separations (24, 25). However, in those methods one component of the binary solvent system contained UV-absorbing material, and recovery of the peptides required destructive analysis of a portion of the effluent. In one case, between 50 and 100% of the sample was required for the purpose of peptide detection using a fluorescamine monitoring system (24).

Our procedure permits the nondestructive detection of peptides on as little as 50 ng of sample compared to 10 ng for destructive methods using fluorescamine or *o*-phthalaldehyde (25). Sample recovery can be accomplished by evaporation or lyophilization and in no case has the procedure altered or destroyed any of the amino acids found in the peptides. This makes HPLC purification of peptides an ideal preparative step prior to sequence analysis.

**Acknowledgments**—We thank Dr. Peter E. Nute for providing both the fetal globin samples and the quantitative data on the percentage of  $^G\gamma$  chains present in the  $\gamma$ CB3 samples. We also thank Dr. Fred E. Regnier for helpful discussions during the course of this work.

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