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SEPARATION OF TRYPTIC PEPTIDES OF NORMAL AND ABNORMAL α, β, γ, AND δ HEMOGLOBIN CHAINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography (HPLC) was used to separate tryptic peptides of the normal α, β, γ, and δ chains of human hemoglobins A, F, and A_2 and of the abnormal chains of 25 hemoglobin variants. In addition, the separation of chymotryptic peptides of the oxidized core of the normal α chain by HPLC was evaluated. HPLC has several advantages over conventional methods used for the separation of proteolytic fragments of hemoglobin chains. The method is fast, and reproducible, and requires only small quantities of material. Several peptides are eluted as single zones, thus eliminating the need of rechromatography for further purification. Characteristic changes in the elution pattern of the peptides often indicate specific modifications.

INTRODUCTION

Structural characterization of the many human hemoglobin (Hb) variants is usually based on the analyses of tryptic (or chymotryptic) peptides of isolated α, β, γ, δ, or hybrid (γβ, βδ, δβ) chains being separated by one of many different procedures. The most popular methods include a combination of chromatography and electrophoresis on paper, cellulose, or on silica gel thin-layer plates (known as the fingerprinting technique), macro-chromatography on cation exchangers (Bio-Rad Aminex A-5, Spinco PA-35, Technicon Chromobead type P, Dowex 50-X2, 50-X4, 50-X8) followed by rechromatography of selected peptides by anion-exchange chromatography (Dowex 1-X2), and microchromatography on columns of PA-35 ion-exchange resin introduced by Jones. References to these methods can be found in a recent monograph[1].

The development of high-performance liquid chromatographic procedures (HPLC)[2–4] offers a new approach to separate micro quantities of tryptic peptides of

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the various hemoglobin chains within a few hours, thus facilitating the identification of numerous hemoglobin variants. This possibility was explored through analyses of normal α, β, γ, and δ chains and of the abnormal chains of 25 different hemoglobin variants. The data indicated that HPLC often offers distinct advantages over more conventional procedures of peptide separation.

MATERIALS AND METHODS

Materials

The normal hemoglobins A, A_2, and F were obtained from one of the authors (T.H.J.H.) and from the cord blood sample of a normal baby. The hemoglobins were isolated by DEAE-cellulose chromatography[5]. In addition, seven α chain variants, thirteen β chain variants, one γ chain variant, two δ chain variants, and two hybrid hemoglobins were studied (Table I). These abnormal hemoglobins were also isolated by DEAE-cellulose column chromatography. The polypeptide chains of the normal hemoglobins and the hemoglobin variants were separated by CM-cellulose chromatography as described by Clegg *et al.*[6]. Isolated β, γ, δ, and hybrid chains were amino-ethylated (AE) by the method of Jones[7] prior to tryptic digestion. The normal and abnormal α chains were studied without prior modification. The insoluble core of the α chain tryptic digest was isolated at pH 6.5, oxidized with performic acid, and next digested with chymotrypsin; the methodology is detailed in ref. 1.

TABLE I

GLOSSARY OF ABNORMAL HEMOGLOBINS USED IN THIS STUDY

Identification of these variants is based on data supplied by suppliers of the variants or by data obtained by the authors using conventional procedures[1].

Variant	*Substitution*	*Reference*	*Variant*	*Substitution*	*Reference*
I-Interlaken	α 15 Gly → Asp	8	Tacoma	β 30 Arg → Ser	22
I-Philadelphia	α 16 Lys → Glu	9	Austin	β 40 Arg → Ser	23
G-Montgomery	α 48 Leu → Arg	10	Malmö	β 97 His → Gln	24
J-Sardegna	α 50 His → Asp	11	P-Galveston	β117 His → Arg	25
G-Russ	α 51 Gly → Arg	12	Riyadh	β120 Lys → Asn	26
Shimonoseki	α 54 Gln → Arg	13	Hacettepe	β127 Gln → Glu	27
Suresnes	α141 Arg → His	14	F-Malta-I	γ117 His → Arg	28
S	β 6 Glu → Val	15			
G-San Jose	β 7 Glu → Gly	16	A_2' (= B_2)	δ 16 Gly → Arg	29
Saki	β 14 Leu → Pro	17	A_2-Flatbush	δ 22 Ala → Glu	30
J-Georgia	β 16 Gly → Asp	18			
Alamo	β 19 Asn → Asp	19	Lepore-Washington	$\delta\beta$hybrid	31
Connecticut	β 21 Asp → Gly	20	P-Nilotic	$\beta\delta$hybrid	32
E	β 26 Glu → Lys	21			

Some 5 to 10 mg α chain, or AE-β, AE-γ, or AE-δ chain were dissolved in 10 ml water and digested under constant stirring for 4 h at 22–25° with TPCK- trypsin (Worthington, Freehold, N.J., U.S.A.) at pH 8.5–8.9 (6 mg ammonium bicarbonate was dissolved in the Hb solution, the pH was adjusted with 0.5 *M* NaOH to 8.9, and

0.1–0.2 mg trypsin in 0.2–0.3 ml 0.001 *M* HCl was added). Next the pH of the α chain digest was adjusted with dilute HCl to 6.3–6.5 and the insoluble core removed by centrifugation. The pH of the clear supernatant of the α chain digest (and also that of the AE-β, AE-γ, and AE-δ chain digests) was adjusted to 2.5–3.0 whereafter the solution was lyophilized. Some 2–3 mg digest was dissolved in 100 μl of 10% acetic acid and centrifuged for 10 min at 3600 rpm in an Adams Sero Fuge (Clay Adams, Parsippany, N.J., U.S.A.). Nearly the entire supernatant was applied to the column (μBondapak C_{18}, part No. 27324, Waters Assoc., Milford, Mass., U.S.A.) of the HPL chromatograph (Perkin Elmer Series 3 Microprocessor with LC-55 UV detector and LKB Model 2070 Ultrarac II fraction collector). The solvents used were (A) 50% acetonitrile in 0.01 *M* ammonium acetate, pH 5.7; and (B) 0.01 *M* ammonium acetate, pH 5.7. The most suitable solvent program was T-1, 120 min (1%–60%) gradient curve 1; T-P, 20 min (100%). Chart speed: 30 cm/h; flow-rate: 1.5 ml/min at room temperature; wavelength: 220 nm; range: 0.05 absorbance units full scale. The reagents were analytical grade. Acetonitrile was obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.), high purity grade and distilled in glass, and was used without further purification. The ammonium acetate solution was prefiltered through a millipore Type HA 0.45 μm filter. All solvents were degassed prior to use.

Other procedures

Each isolated zone was dried under nitrogen and hydrolyzed in 6 *M* HCl with 9 mg/dl phenol at 110° for 24 h. The resulting hydrolysate was analyzed with a Beckman 121M amino acid analyzer equipped with a system AA computing integrator.

RESULTS AND DISCUSSION

The normal α chain and several α chain variants

Fig. 1 shows the chromatogram of the tryptic peptides of the normal non-aminoethylated α chain. Most of the fragments were isolated as single, relatively pure zones. Peptides T-5 and T-6 were partially separated, and peptides T-1 and T-2, T-10 and T-11, and T-8 and T-9 were in part recovered as larger fragments, T-1, 2, T-10,11, and T-8,9, respectively. The small peptides T-7 (Gly-His-Gly-Lys) and T-8 (Lys) were the first to be eluted immediately followed by a mixture of the peptides T-2 (Thr-Asn-Val-Lys) and T-10 (Leu-Arg), and next by T-14 (Tyr-Arg). Several attempts were made to improve the separation of these five peptides, but failed. The peptides T-5 and T-6 could be separated completely from each other through a small change in the gradient (see ref. 33) which, however, decreased the resolution of some of the other peptides. Table II lists the amino acid compositions of all fragments; the data indicate a reasonable purity for several peptides, allowing an evaluation of their respective structures.

The seven α chain variants included in this study concerned two with substitutions in peptide T-3, four with substitutions in peptide T-6, and one with a substitution in T-14 (Table I and Fig. 1). The substitution in Hb I-Interlaken results in an abnormal T-3 which exhibits a slight difference in mobility, but that in Hb I-Philadelphia causes the elimination of the T-3 and T-4 peptides from the chromatogram because these fragments are recovered as one single T-3,4 peptide which is well separated from all other fragments. Amino acid analyses are given in Table III. The

TABLE II

THE AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF THE NON-AMINOETHYLATED *α* CHAIN, SEPARATED BY HPLC

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide of the normal *α* chain. The presence of tryptophan is indicated by a + sign.

	T-1	*T-2; T-10**	*T-1,2*	*T-3*	*T-4*	*T-5***	*T-6***	*T-7; T-8**	*T-9*	*T-8,9*	*T-11*	*T-10,11*	*T-14*
Lysine	1.00 (1)	1.00 (1)	2.00 (2)	1.00 (1)		0.93 (1)	1.05 (1)	1.90 (2)	1.06 (1)	1.75 (2)	1.00 (1)	1.00 (1)	
Histidine					1.06 (1)		1.59 (2)	0.97 (1)	2.59 (3)	2.82 (3)			
Arginine		1.00 (1)			1.00 (1)							0.90 (1)	1.00 (1)
Aspartic acid	0.97 (1)	1.08 (1)	2.38 (2)				1.33 (1)		6.11 (6)	6.02 (6)	2.04 (2)	1.96 (2)	
Threonine		1.11 (1)	1.00 (1)			1.76 (2)	1.04 (1)		1.00 (1)	0.95 (1)			
Serine	0.97 (1)		1.02 (1)			1.18 (1)	2.08 (2)		2.21 (2)	1.95 (2)			
Glutamic acid					2.68 (3)		1.24 (1)						
Proline	1.04 (1)		1.07 (1)			0.97 (1)	1.05 (1)		1.01 (1)	1.00 (1)	1.07 (1)	0.86 (1)	
Glycine				1.00 (1)	2.86 (3)		1.27 (1)	2.13 (2)					
Alanine	1.04 (1)		1.08 (1)	1.97 (2)	4.46 (4)		0.86 (1)		6.90 (7)	7.05 (7)			
Valine	0.79 (1)	0.95 (1)	1.58 (2)		1.10 (1)		1.15 (1)		2.15 (3)	2.64 (3)	2.01 (2)	2.00 (2)	
Methionine						0.53 (1)			0.83 (1)	+ (1)			
Leucine	0.71 (1)	0.96 (1)	0.67 (1)		0.97 (1)	1.00 (1)	1.04 (1)		3.32 (4)	3.70 (4)		1.06 (1)	0.79 (1)
Tyrosine					0.71 (1)		1.04 (1)						
Phenylalanine						1.74 (2)	1.73 (2)				1.01 (1)	1.07 (1)	
Tryptophan				+ (1)									

* These peptides were not separated from each other (see Fig. 1); their quantities isolated from the chromatogram were about the same.

** These peptides were partially overlapping; the analyses are from the front and tail segments of the appropriate zone (see Fig. 1).

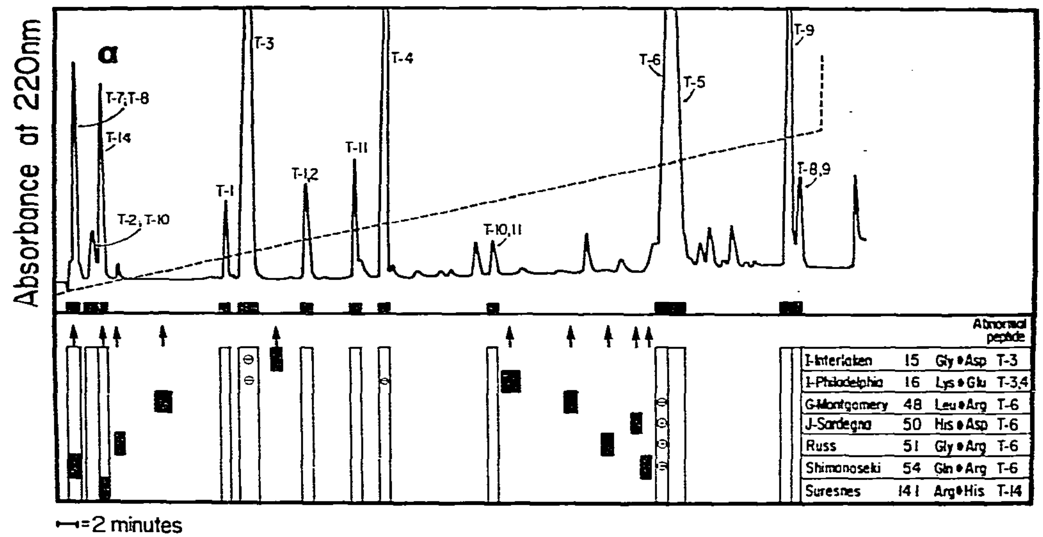

Fig. 1. Separation of peptides from tryptic digests of normal and abnormal human hemoglobin α chains by HPLC. Top section: Chromatogram of a normal α chain digest. Bottom section: Changes in mobility of selected peptides in chromatograms of digests of different types of α chain. ⊖ denotes that this normally occurring peptide is absent and replaced by one or two others (indicated by ■).

hemoglobins G-Montgomery, J-Sardegna, Russ, and Shimonoseki have substitutions in the C-terminal segment of the T-6 peptide as follows:

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47  48  49  50  51  52  53  54  55  56
Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys
    (Arg)   (Asp)(Arg)      (Arg)
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Three of the four substitutions concern the introduction of an arginyl residue resulting in the appearance of two additional smaller tryptic peptides replacing the normal T-6. These fragments were readily identified (Table III) except the Val-Lys dipeptide which was present in the digest of the α-Shimonoseki chain and was eluted with the first two peptides, T-7 and T-8. The abnormal T-14 peptide (Tyr-His) of Hb-Suresnes had the same mobility as the T-14 peptide (Tyr-Arg) of the normal α chain. This observation indicates that the absence of a distinct change in the elution pattern of the tryptic peptide not necessarily excludes a structural abnormality in any one of these fragments.

The chymotryptic peptides of the oxidized core of the α chain

The α chain core concerns residues 100–139, inclusively; the sequence of this fragment is given in Fig. 2. The α chain was digested for 4 or 24 h with trypsin, and the core isolated at pH 6.4 by centrifugation. The precipitate was washed with small volumes of distilled water, pH 6.5, to remove traces of soluble peptides. Next, the cores were oxidized with performic acid and digested for 4 h with chymotrypsin at room temperature and at pH 9.0. After adjusting the pH to 2.5, the digests were lyophilized, and the chymotryptic peptides separated with the same gradient as used for the soluble peptides of the α chain.

The chromatogram of the chymotryptic digest of the oxidized core isolated

TABLE III

THE AMINO ACID COMPOSITION OF SELECTED PEPTIDES ISOLATED FROM TRYPTIC DIGESTS OF SEVEN α CHAIN VARIANTS

The data are given in residues/mole peptide. The numbers between parentheses refer to the numbers of amino acid residues present in the corresponding peptides of the α chain from Hb A. The presence of tryptophan is indicated by a + sign.

	I-Interlaken		*I-Philadelphia*		*G-Montgomery*			*J-Sardegna*		*Russ*			*Shimonoseki**				*Suresnes*	
	T-3	*(T-3)*	*T-3,4*	*(T-3,4)*	*T-6^A*	*T-6^B*	*(T-6)*	*T-6*	*(T-6)*	*T-6^A*	*T-6^B*	*(T-6)*	*T-6^A*	*T-6^B* + *T-7*	*(T-6)*	*(T-7)*	*T-14*	*(T-14)*
Lysine	1.00	(1)	0.0	(1)		0.87	(1)	1.00	(1)		1.00	(1)		1.78	(1)	(1)		
Histidine			0.95	(1)	1.09	1.04	(2)	0.97	(2)	1.70		(2)	1.31	0.92	(2)	(1)	1.05	
Arginine			1.00	(1)	0.89					1.00			1.00				0	(1)
Aspartic acid	0.90				1.00		(1)	1.89	(1)	1.08		(1)	0.56		(1)			
Threonine					0.82		(1)	1.04	(1)	0.98		(1)	1.02		(1)			
Serine						1.93	(2)	1.50	(2)	1.02	1.05	(2)	2.04		(2)			
Glutamic acid			3.70	(3)		1.00	(1)	1.06	(1)		1.00	(1)	0.08		(1)			
Proline					0.80		(1)	1.14	(1)	1.04		(1)	0.95		(1)			
Glycine	0.06	(1)	3.48	(4)		1.20	(1)	1.16	(1)	0.12		(1)	1.22	2.18	(1)	(2)		
Alanine	1.89	(2)	6.52	(6)		1.09	(1)	1.13	(1)		1.08	(1)	1.49		(1)			
Valine			1.23	(1)		0.82	(1)	1.11	(1)		0.80	(1)		1.12	(1)			
Leucine			1.10	(1)	0		(1)	1.08	(1)	0.92		(1)	0.75		(1)			
Tryosine			0.86	(1)	0.98		(1)	0.79	(1)	0.63		(1)	0.89		(1)		0.94	(1)
Phenylalanine					2.07		(2)	1.49	(2)	1.75		(2)	2.09		(2)			
Tryptophan	+	(1)	+	(1)														

* Peptide T-6^B was contaminated with normal peptide T-7 (see Fig. 1).

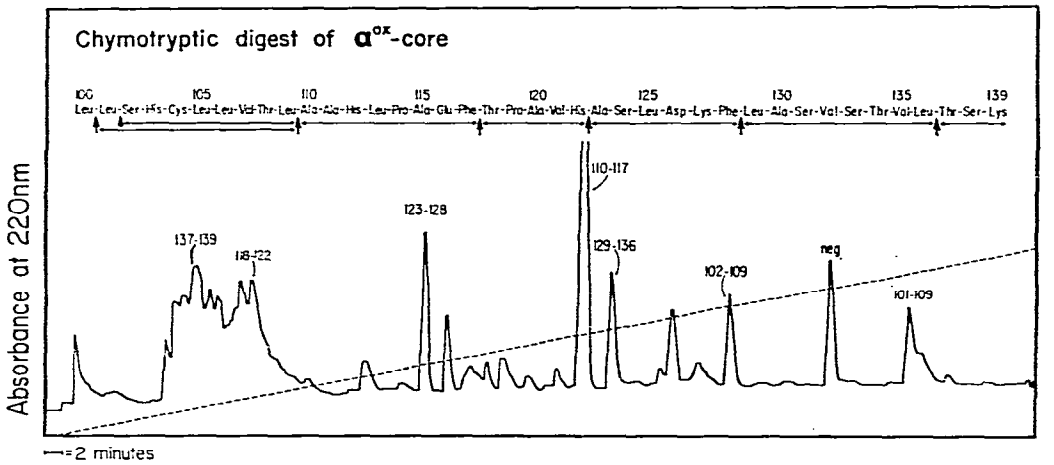

Fig. 2. HPLC separation of peptides from a chymotryptic digest of the oxidized core of a normal human hemoglobin α chain. The sequence of the core is listed at the top of the figure. The numbers identifying the various zones refer to positions in the intact α chain.

from the 4-h tryptic digest resulted in numerous major and minor zones containing many fragments of soluble peptides mixed with chymotryptic peptides of the core. However, the chymotryptic digest of the oxidized core from a 24-h tryptic digest resulted in a much more acceptable chromatogram containing mostly the major core peptides which were well separated from each other except for the zones in the early part of the chromatogram (Fig. 2). The amino acid compositions of the fragments are listed in Table IV; the data indicate an acceptable purity for each of these peptides.

TABLE IV

THE AMINO ACID COMPOSITION OF CHYMOTRYPTIC PEPTIDES OF THE OXIDIZED CORE OF A NORMAL α CHAIN SEPARATED BY HPLC

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide.

	*137–139**	*118–122*	*123–128*	*110–117*	*129–136*	*102–109*	*101–109*
Lysine	1.00 (1)		0.97 (1)				
Cysteic acid						0.80 (1)	+ (1)
Histidine		1.00 (1)		0.72 (1)		0.94 (1)	0.85 (1)
Aspartic acid			1.08 (1)				
Threonine	0.80 (1)	1.18 (1)			0.83 (1)	0.99 (1)	0.90 (1)
Serine	0.99 (1)		0.98 (1)		1.50 (2)	1.00 (1)	1.00 (1)
Glutamic acid				1.30 (1)			
Proline		1.12 (1)		1.04 (1)			
Alanine		0.95 (1)	1.00 (1)	2.52 (3)	1.00 (1)		
Valine		0.96 (1)			1.76 (2)	1.09 (1)	1.01 (1)
Leucine			1.01 (1)	0.90 (1)	1.94 (2)	2.27 (3)	2.63 (4)
Phenylalanine			0.89 (1)	1.00 (1)			

* Position numbers in the intact α chain; the peptides are listed in order of elution (see Fig. 2).

TABLE V

THE AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF THE AMINOETHYLATED β CHAIN, SEPARATED BY HPLC

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide of the normal β chain. The presence of tryptophan is indicated by a + sign.

	T-1	*T-2*	*T-3*	*T-4*	*T-5*	*	*(T-6)*	*(T-7)*	*(T-8)*	*T-9*	*T-10*	*T-11*	*T-12A*	*T-12B*	*T-13*	*T-14*	*T-15*
Lysine	1.00 (1)	0.90 (1)			1.00 (1)	3.00 (1)		(1)	(1)	1.00 (1)	1.00 (1)			1.00 (1)	1.03 (1)	1.00 (1)	
AE-Cysteine											0.56 (1)		1.00 (1)				
Histidine	0.62 (1)					1.16		(1)		0.87 (1)	0.65 (1)	0.98 (1)		1.77 (2)		0.96 (1)	1.00 (1)
Arginine			1.00 (1)	0.87 (1)								1.00 (1)					
Aspartic acid			1.90 (2)		3.09 (3)					3.00 (3)	1.01 (1)	2.16 (2)	0.97 (1)			1.46 (1)	
Threonine	1.06 (1)	0.91 (1)		0.97 (1)	0.80 (1)						1.33 (2)				1.00 (1)		
Serine		0.96 (1)			1.70 (2)					0.91 (1)	0.89 (1)						
Glutamic acid	2.00 (2)		2.05 (2)	1.00 (1)	1.10 (1)						1.00 (1)	1.05 (1)			2.96 (3)		
Proline	0.98 (1)			1.01 (1)	1.75 (2)							1.07 (1)			2.12 (2)		
Glycine		1.08 (1)	3.00 (3)		2.09 (2)	1.19		(1)		1.77 (2)	1.09 (1)		1.02 (1)	1.22 (1)		1.11 (1)	
Alanine		2.05 (2)	1.04 (1)		1.12 (1)	1.00		(1)		1.82 (2)	1.10 (1)			1.29 (1)	2.00 (2)	4.40 (4)	
Valine	0.66 (1)	0.99 (1)	3.28 (3)	1.06** (2)	1.24 (1)	1.00	(1)			1.00 (1)		1.13 (1)	1.84 (2)	1.09 (1)	1.00 (1)	2.40** (3)	
Methionine					0.29 (1)												
Leucine	0.91 (1)	1.00 (1)	0.86 (1)	2.20 (2)	1.26 (1)					3.51 (4)	2.02 (2)	1.05 (1)	2.73 (3)	0.98 (1)		1.00 (1)	
Tyrosine				0.90 (1)											0.96 (1)		0.88 (1)
Phenylalanine					2.55 (3)					0.83 (1)	0.94 (1)	1.00 (1)		0.99 (1)	1.02 (1)		
Tryptophan		+ (1)		+ (1)													

* Mixture of T-6, T-7 and T-8 (see Fig. 3).

** Low value in 24-h hydrolysate due to Val-Val bond.

The normal AE-β chain and several β chain variants

Nearly all tryptic peptides of the AE-β chain digest were separated from each other (Fig. 3), and their amino acid compositions indicated an acceptable purity for most of these fragments (Table V). Peptides T-6 (Val-Lys), T-7 (Ala-His-Gly-Lys), and T-8 (Lys) were eluted as one single zone in the first few tubes of chromatogram. Peptide T-5 occurred in two zones, which is probably due to oxidation of the methionyl residue. Peptide T-9 was also recovered as the double peptide T-8,9 which contained one extra lysyl residue and eluted behind the T-9 peptide.

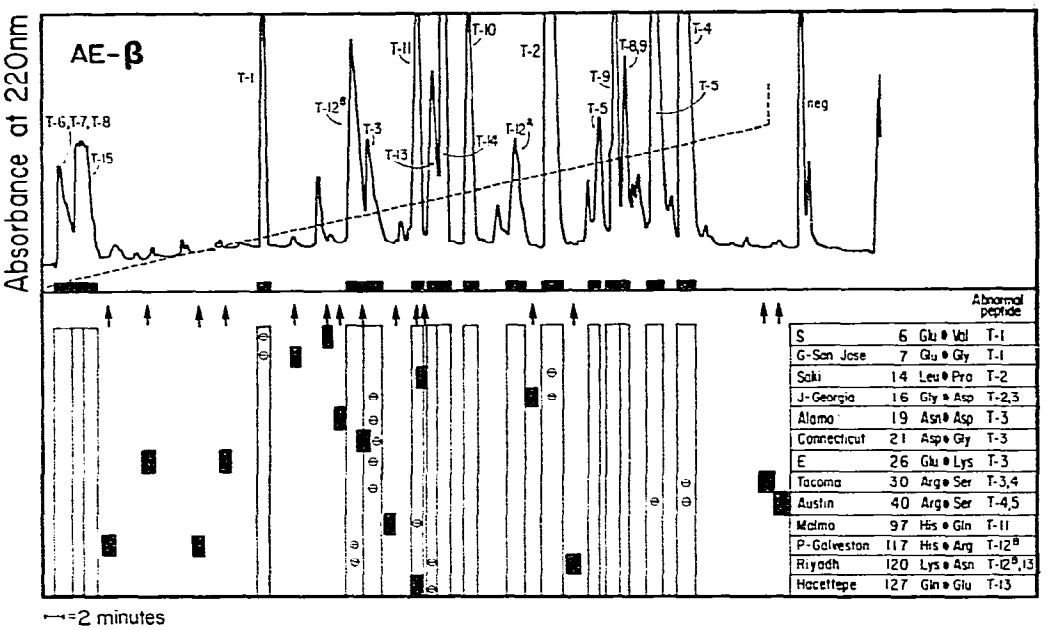

Fig. 3. Separation of peptides from tryptic digests of the aminoethylated normal and abnormal human hemoglobin β chains by HPLC. Top section: Chromatogram of a normal β chain digest. Bottom section: Changes in mobility of selected peptides in chromatograms of digests of 13 types of β chain. ⊖ denotes the absence of the normally occurring peptide which is replaced by one or two others (indicated by ■).

Fig. 3 also lists the thirteen β chain variants that were studied with this procedure, while Tables VI and VII list the amino acid compositions of the appropriate abnormal peptides. The two variants with a substitution of a residue in peptide T-1 were readily identified because the abnormal peptides eluted as single zones, and their amino acid compositions clearly indicated the type of substitution. The two variants with a variation in peptide T-2 (Hb Saki and Hb J-Georgia) were also readily identified, although the abnormal T-2 peptide isolated from the β-Saki chain was contaminated with peptide T-11. The double peptide T-2,3 which is the result of a Gly → Asp substitution at the penultimate position of the T-2 peptide was isolated as a single zone while the T-2 and T-3 peptides had disappeared from the chromatogram. Of the four variants with substitutions of a residue in the T-3 peptide, three were readily identified (Alamo, E, and Tacoma), but the abnormal T-3 peptide of Hb Connecticut separated only partially from the T-12^{B} peptide (Fig. 3

TABLE VI

THE AMINO ACID COMPOSITION OF SELECTED PEPTIDES ISOLATED FROM TRYPTIC DIGESTS OF SEVEN β CHAIN VARIANTS

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues present in the corresponding peptides of the β chain from Hb A. The presence of tryptophan is indicated by a + sign.

	S		*G-San Jose*		*Saki*			*J-Georgia*		*Alamo*		*Connecticut*			*E*		
	T-1	*(T-1)*	*T-1*	*(T-1)*	*T-2***	*(T-2)*	*(T-11)*	*T-2,3*	*(T-2,3)*	*T-3*	*(T-3)*	*T-3***	*(T-3)*	*(T-12^B)*	*T-3^A*	*T-3^B*	*(T-3)*
Lysine	1.01	(1)	0.89	(1)	1.00	(1)		1.00	(1)			0.34		(1)	0.73		
AE-Cysteine																	
Histidine	0.60	(1)	0.97	(1)	0.11		(1)					0.58		(2)			
Arginine					0.13		(1)	0.75	(1)	1.00	(1)	1.00	(1)			1.00	(1)
Aspartic acid					0.22		(2)	2.57	(2)	1.14	(2)	1.07	(2)		1.71		(2)
Threonine	1.00	(1)	0.92	(1)	1.16	(1)		0.95	(1)								
Serine					0.85	(1)		1.14	(1)								
Glutamic acid	0.98	(2)	1.08	(2)	0.15		(1)	1.75	(2)	2.28	(2)	2.02	(2)		1.02		(2)
Proline	1.22	(1)	1.18	(1)	1.18		(1)										
Glycine			1.09		1.01	(1)		2.70	(4)	3.25	(3)	4.02	(3)	(1)	1.65	0.77	(3)
Alanine					1.52	(2)		2.67	(3)	1.45	(1)	1.34	(1)	(1)		0.64	(1)
Valine	1.67	(1)	0.90	(1)	1.03	(1)	(1)	3.28	(4)	2.83	(3)	2.98	(3)	(1)	3.00		(3)
Methionine																	
Leucine	0.88	(1)	1.00	(1)	0.11	(1)	(1)	1.79	(2)	1.16	(1)	1.01	(1)	(1)		0.71	(1)
Tyrosine																	
Phenylalanine					0.10		(1)					0.38		(1)			
Tryptophan					+	(1)		+	(1)								

* Contaminated.

TABLE VII

THE AMINO ACID COMPOSITION OF SELECTED PEPTIDES ISOLATED FROM TRYPTIC DIGESTS OF SIX ADDITIONAL β CHAIN VARIANTS

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues present in the corresponding peptides of the β chain from Hb A. The presence of tryptophan is indicated by a + sign.

	Tacoma		*Austin*		*Malmö*		*P-Galveston*				*Riyadh*		*Hacettepe*			
	T-3,4	*(T-3,4)*	*T-4,5*	*(T-4,5)*	*T-11*	*(T-11)*	*T-12^{A}*	*T-12^{B}*	*T-12^{C}*	*(T-12)*	*T-12^{B},13*	*(T-12^{B},13)*	*T-11**	*(T-11)*	*T-13**	*(T-13)*
Lysine			1.22	(1)					0.90	(1)	1.00	(2)			1.00	(1)
AE-Cysteine							1.00			(1)						
Histidine					0.26	(1)		0.95		(2)	2.06	(2)	0.92	(1)		
Arginine	0.97	(2)	0.28	(1)	0.99	(1)		1.00					1.00	(1)		
Aspartic acid	1.80	(2)	2.98	(3)	1.82	(2)	0.99			(1)	0.90		2.03	(2)		
Threonine	1.01	(1)	2.00	(2)							1.40	(1)			1.15	(1)
Serine	1.20		2.57	(2)												
Glutamic acid	2.48	(3)	2.43	(2)	1.92	(1)					2.78	(3)	0.97	(1)	2.45	(3)
Proline	1.00	(1)	2.44	(3)	1.10	(1)					1.98	(2)	1.10	(1)	2.24	(2)
Glycine	2.93	(3)	1.72	(2)			1.00		1.22	(2)	1.10	(1)				
Alanine	0.93	(1)	1.00	(1)				0.88		(1)	3.16	(3)			2.25	(2)
Valine	3.53**	(5)	2.50**	(3)	0.83	(1)	2.09	0.91		(3)	1.68	(2)	1.04	(1)	1.04	(1)
Methionine			+	(1)												
Leucine	3.22	(3)	3.12	(3)	0.97	(1)	3.00	1.00		(4)	0.72	(1)	0.93	(1)		
Tyrosine	0.92	(1)	0.74	(1)							0.92	(1)			0.83	(1)
Phenylalanine			1.67	(3)	1.00	(1)			0.86	(1)	1.83	(2)	1.00	(1)	0.83	(1)
Tryptophan			+	(1)												

* Mixtures of about equal amounts of T-11 and T-13.

** Low value due to presence of Val-Val bond.

and Table VI). The double peptide T-3,4 found in Hb Tacoma (30 Arg → Ser) was isolated from the last part of the chromatogram while the T-3 and T-4 zones were absent. A similar situation was observed for Hb Austin (40 Arg → Ser): the double peptide T-4,5 again was eluted as the last peptide while the T-4 and T-5 peptides were not observed. The amino acid composition of the T-3,4 peptide of Hb Tacoma was most reasonable, but that of the T-4,5 peptide of Hb Austin was far less satisfactory which, however, did not prevent the identification of the appropriate substitution (Table VII). The substitutions in Hb Malmö, P-Galveston, Riyadh, and Hacettepe were also readily identified. The abnormal T-11 of Hb Malmö eluted slightly ahead of the normal T-11 peptide. The His → Arg substitution in Hb P-Galveston resulted in two new peptides (Phe-Gly-Lys and Val-Leu-Ala-His-Arg) which eluted in that order between the T-15 and T-1 peptides. The replacement of lysyl residue of T-12^{B} by an asparaginyl residue in Hb Riyadh eliminated T-12^{B}. A double peptide (T-12^{B},13) was eluted in front of and well separated from the normal T-10 peptide. The Gln → Glu substitution in the β chain of Hb Hacettepe changed the chromatographic mobility of the T-13 peptide slightly; unfortunately, the abnormal T-13 eluted together with the normal T-11 fragment (Table VII).

The normal AE-γ chain and one γ chain variant

The bottom section of Fig. 4 illustrates that most tryptic peptides of the AE-γ chain can be separated by HPLC. The chromatogram requires several comments. *First*, peptides T-6, T-7, and T-8 are eluted as a single zone, similarly as seen for the digest of the AE-β chain and also for that of the AE-δ chain (Fig. 4, top section). *Second*, peptide T-5 which is eluted last, is not always recovered from each AE-γ chain digest; the reason for this behavior is not understood. *Third*, the γ chain of Hb F usually is a mixture of two different species which differ at position 136 because this position can be occupied by either a glycyl or an alanyl residue[34]. The heterogeneity results in the existence of two T-15 tryptic peptides (Met-Val-Thr-*Gly* (or *Ala*)-Val-Ala-Ser-Ala-Leu-Ser-Ser-Arg) which can be separated by HPLC. The $^{A}\gamma$T-15 is eluted as a single zone, while the $^{G}\gamma$T-15 is only partially resolved from the T-9 peptide. *Fourth*, the replacement of isoleucyl residue in position 75 was first discovered in the Hb variant Hb F-Sardinia[35], has since then been observed in numerous other fetal Hb samples[36–38]. Convincing evidence has been presented indicating that this variation occurs in the $^{A}\gamma$ chain, and thus should be identified as the $^{A}\gamma^{T}$ chain[37–39]. Tryptic peptides $^{I}\gamma$T-9 (Val-Leu-Thr-Ser-Leu-Gly-Asp-Ala-*Ile*-Lys) and $^{T}\gamma$T-9 (Val-Leu-Thr-Ser-Leu-Gly-Asp-Ala-*Thr*-Lys) can readily be separated by HPLC. The $^{I}\gamma$T-9 fragment eluted together with the γT-11 peptide, while the $^{T}\gamma$T-9 eluted as a single zone in front of the T-14 peptide (the γ chain used in this study did not contain the $^{A}\gamma^{T}$ chain and thus no $^{T}\gamma$T-9 peptide was recovered). These distinct separations offer unique opportunities to study the occurrence of the $^{G}\gamma^{I}$, $^{A}\gamma^{I}$, and $^{A}\gamma^{T}$ chains in isolated fetal hemoglobins. The separation of the $^{G}\gamma$T-15 and $^{A}\gamma$T-15 peptides by HPLC was the basis of a new procedure to determine the ratio of the $^{G}\gamma$ and $^{A}\gamma$ chains in the Hb F that is synthesized *in vivo* by red cell precursors during incubation in an appropriate medium containing [^{35}S]methionine[40,41]. *Fifth*, only small amounts of the T-13 peptide were recovered, and a satisfactory amino acid analysis was not obtainable.

Table VIII lists the results of the amino acid analyses. Some of the peptides

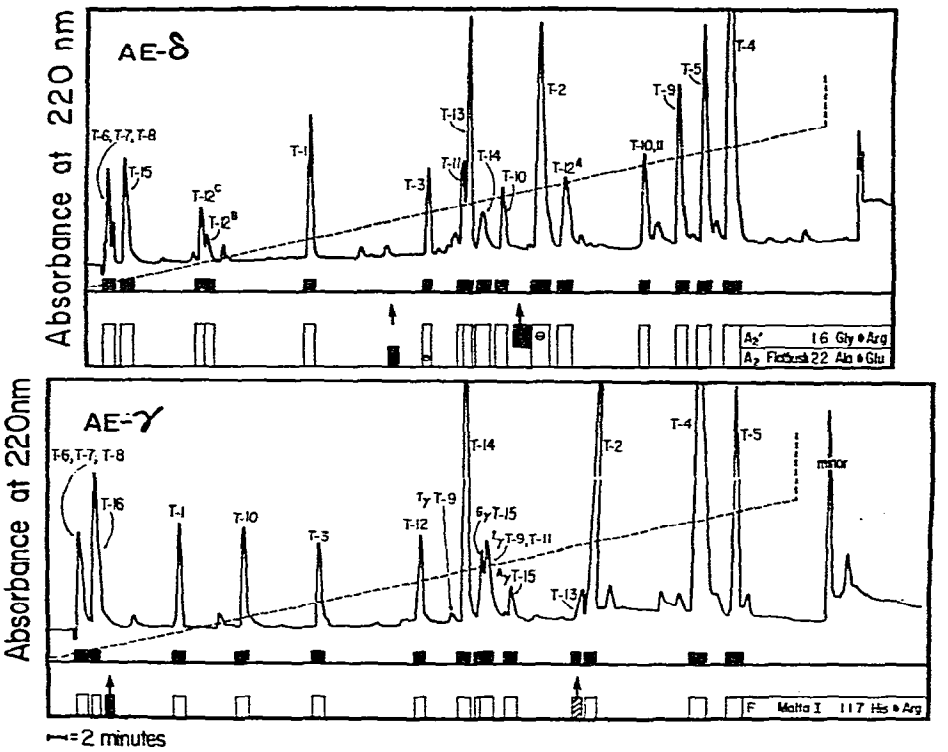

Fig. 4. Separation of peptides from tryptic digests of the aminoethylated human δ chain (top section) and the human γ chain (bottom section) by HPLC.

(T-1, T-3, T-4, T-10, T-12, T-14, T-16) were obtained as pure components, others (T-2, T-5, $^{G}\gamma$T-15, $^{A}\gamma$T-15) gave somewhat less satisfactory analyses, while the T-6, T-7, and T-8 peptides and also the $^{I}\gamma$T-9 and T-11 peptides were eluted as mixtures of about equal molar quantities. An acceptable analysis of T-13 was not obtained.

The variant Hb F-Malta-I has a substitution of histidinyl residue in position 117 by an arginyl residue which results in the occurrence of two fragments, T-13^{A} and T-13^{B}. The larger T-13^{A} fragment likely eluted at the position of the normal T-13 peptide, but again its recovery was very low. The T-13^{B} fragment (Phe-Gly-Lys) was readily recovered from the first part of the chromatogram (for analysis, see Table X).

The normal AE-δ chain and two δ chain variants

Fig. 4, top section, illustrates that most tryptic peptides of the AE-δ chain were eluted as single zones, excluding the peptides T-6, T-7, and T-8. With only a few exceptions (T-10, T-12^{A}) satisfactory analyses were obtained (Table IX). Peptide T-12 (residues 105-120, inclusive) was recovered as three small fragments because of the aminoethylated cysteinyl residue in position 112, and an arginyl residue located in position 116. The two extra peptides, T-12^{B} (Val-Leu-Ala-Arg) and T-12^{C} (His-Phe-Gly-Lys), were eluted in reverse order between peptides T-15 and T-1.

Identification of the two δ chain variants provided no difficulties. The abnormal

TABLE VIII

THE AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF THE AMINOETHYLATED γ CHAIN, SEPARATED BY HPLC

The data are given in residues/mole peptide.The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide of the normal γ chain. The presence of tryptophan is indicated by a + sign.

	T-1	*T-2*	*T-3*	*T-4*	*T-5*	*	*T-6*	*T-7*	*T-8*
Lysine	1.00 (1)	1.00 (1)			1.00 (1)	3.00	(1)	(1)	(1)
AE-Cysteine									
Histidine	0.92 (1)					1.00		(1)	
Arginine			1.00 (1)	1.00 (1)					
Aspartic acid	1.04 (1)		2.01 (2)		3.19 (3)				
Threonine	1.00 (1)	1.68 (2)	1.00 (1)	1.02 (1)					
Serine		1.19 (1)			3.59 (4)				
Glutamic acid	2.03 (2)		1.92 (2)	1.06 (1)					
Proline				0.88 (1)	0.63 (1)				
Glycine	0.94 (1)	1.43 (1)	3.12 (3)		2.29 (2)	1.06		(1)	
Alanine		1.10 (1)	1.01 (1)		2.07 (2)	0.94		(1)	
Valine			2.01 (2)	1.38[§] (2)		1.00	(1)		
Methionine					+ (1)				
Isoleucine		0.61 (1)			0.68 (1)				
Leucine		1.16 (1)	0.94 (1)	1.74 (2)	1.13 (1)				
Tyrosine				0.79 (1)					
Phenylalanine	0.94 (1)				2.76 (3)				
Tryptophan		+ (1)		+ (1)					

* Mixture of T-6, T-7 and T-8 (see Fig. 4).
** Mixture of $^{I}\gamma$T-9 and T-11.
*** Quantity too low for accurate analysis.
[§] Value is low in 24-h hydrolysate due to Val-Val bond.

T-2 peptide of Hb A_2' moved slightly ahead of the normal δT-2 peptide while the free lysyl residue (residue 17 of the δ chain) resulting from the Gly → Arg substitution at position 16 of the δ-A_2' chain eluted with the T-6, T-7, and T-8 mixture. The single Ala → Glu substitution at position 22 in Hb A_2-Flatbush resulted in a change in chromatographic mobility of the T-3 peptide allowing an easy identification. Amino acid data are given in Table X. It appears that micro HPLC is ideally suited for the identification of δ chain variants also because often only a small quantity of the abnormal chain is available for structural analyses.

The $\delta\beta$ chain of Hb Lepore-Washington and the $\beta\delta$ chain of Hb P-Nilotic (for references, see ref. 1)

Differences between the β and δ chain concern ten amino acid residues in various positions as follows (β → δ replacements):

Peptide T-2 (residues 9-17, inclusive) positions 9 (Ser → Thr); 12 (Thr → Asn).
Peptide T-3 (residues 19-30, inclusive) position 22 (Glu → Ala)
Peptide T-5 (residues 41-59, inclusive) position 50 (Thr → Ser)
Peptide T-10 (residues 83-95, inclusive) positions 86 (Ala → Ser); 87 (Thr → Glu).
Peptide T-12 (residues 105-120, inclusive) positions 116 (His → Arg); 117 (His → Asn).

**	$^{I}\gamma$T-9	T-11	T-10	T-12	T-13	T-14	$^{G}\gamma$T-15	$^{A}\gamma$T-15	T-16
1.70	(1)	(1)	1.00 (1)	1.00 (1)	*** (1)	0.94 (1)			
0.80		(1)							
0.93		(1)	0.67 (1)	0.87 (1)	(1)				1.00 (1)
							1.00 (1)	0.82 (1)	
2.15	(1)	(1)	1.90 (2)	2.00 (2)	(1)				
1.76	(1)	(1)			(1)	1.02 (1)	1.06 (1)	1.00 (1)	
2.41	(1)	(1)				1.14 (1)	3.26 (3)	2.83 (3)	
2.30		(2)		1.11 (1)		3.74 (4)			
				1.03 (1)		0.83 (1)			
2.32	(1)	(1)			(2)		1.20 (1)	0.38 (0)	
2.35	(1)	(1)			(1)	1.23 (1)	2.33 (2)	3.74 (3)	
1.10	(1)			0.94 (1)	(3)	0.96 (1)	2.20 (2)	1.92 (2)	
							+ (1)	+ (1)	
0.85	(1)				(1)				
3.45	(2)	(2)	2.03 (2)	0.90 (1)	(4)		1.00 (1)	1.25 (1)	
									0.80 (1)
1.00		(1)		0.98 (1)	(1)	1.00 (1)			
						+ (1)			

Peptide T-13 (residues 121-132, inclusive) positions 125 (Pro → Gln); 126 (Val → Met).

These substitutions have a distinct effect on the elution profiles of the tryptic peptides of the β and δ chains (Fig. 5). The greatest differences are seen for peptide T-2 (the δT-2 peptide elutes considerably faster than the βT-2 peptide), and for T-12^{B} because the introduction of an arginyl residue results in the presence of two smaller δ chain fragments which are recovered in the first part of the chromatogram. Much smaller chromatographic differences are seen for the other four peptides, and their positions in the chromatogram are not highly characteristic for either one of the two types of polypeptide chain.

The hybrid $\delta\beta$ chain of Hb Lepore-Washington has a δ chain structure from residue 1 through at least residue 87 and a β chain structure from at least residue 116 through residue 146. Thus, a tryptic digest of such a chain contains the β chain peptides T-2, T-3, T-5, and T-10. These peptides were indeed readily observed in the chromatogram of such a digest (Fig. 5). The position of the δT-2 peptide and the absence of the δT-12^{B} and δT-12^{C} peptides were most characteristic and readily identified this polypeptide chain. It appears likely that tryptic digests of the $\delta\beta$ chains of Hb Lepore-Hollandia (cross-over between positions 22 and 50) and of Hb Lepore-Baltimore (cross-over between positions 50 and 86) will have elution profiles rather

TABLE IX

THE AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF THE AMINOETHYLATED δ CHAIN, SEPARATED BY HPLC

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide of the normal δ chain. The presence of tryptophan is indicated by a + sign.

	T-1	*T-2*	*T-3*	*T-4*	*T-5*	*	*T-6*	*T-7*	*T-8*
Lysine	1.00 (1)	0.96 (1)			1.00 (1)	3.00	(1)	(1)	(1)
AE-Cysteine									
Histidine	0.88 (1)					0.87		(1)	
Arginine			0.96 (1)	1.00 (1)					
Aspartic acid		0.98 (1)	2.09 (2)		2.90 (3)				
Threonine	1.00 (1)	0.93 (1)		0.94 (1)					
Serine					2.11 (3)				
Glutamic acid	1.57 (2)		1.00 (1)	1.06 (1)	1.01 (1)				
Proline	1.14 (1)			1.06 (1)	1.96 (2)				
Glycine		0.95 (1)	2.33 (3)		2.08 (2)	0.83		(1)	
Alanine		1.87 (2)	2.00 (2)		1.08 (1)	0.64		(1)	
Valine	0.96 (1)	1.00 (1)	3.02 (3)	1.27** (2)	0.96 (1)	1.90	(1)		
Methionine					0.35 (1)				
Isoleucine									
Leucine	0.96 (1)	1.00 (1)	0.81 (1)	2.07 (2)	1.03 (1)				
Tyrosine				1.00 (1)					
Phenylalanine					2.92 (3)				
Tryptophan		+ (1)		+ (1)					

* Mixture of T-6, T-7 and T-8 (Fig. 4).

** Values are low in 24-h hydrolysate because of Val-Val bond.

similar to that of Hb-Lepore-Washington. Amino acid analyses of appropriate peptides (T-3, T-5, and T-10) will distinguish between the three Lepore hemoglobins.

The $\beta\delta$ chain of the anti-Lepore hemoglobin P-Nilotic has a β chain structure from residue 1 through residue 22, and a δ chain structure from residue 50 through residue 146. The elution profile of the tryptic digest of such a chain (Fig. 5) is characterized by the presence of the two smaller δT-12^B and δT-12^C fragments and the absence of the δT-2 peptide. Amino acid analysis of the remaining peptides T-3, T-5, T-10, and T-13 is required for definite characterization of this polypeptide chain. (The results of the amino acid analyses of the various tryptic fragments of the $\delta\beta$-Lepore chain and the $\beta\delta$-P-Nilotic chain are not presented in detail.)

COMMENTS

The material present in the μBondapak C_{18} (10 μm) columns, the acetonitrile gradient, the high pressure of 7–10 MPa (1000–1500 p.s.i.), and to some extent temperature, flow-rate, and pH influence the separation of compounds of relatively small molecular weight. The resolution is in part based on differences in molecular weight, on the hydrophobic nature of the compounds, and to a much lesser extent on differences in charge. The chromatographic analyses of the tryptic digests of the 25 abnormal hemoglobin chains also exemplify the segeneral rules. For instance, double peptides formed because of specific substitutions are eluted in the later part of the chromatogram (examples are αT-3,4 in Hb I-Philadelphia; αT-3,4 in Hb-Tacoma; βT-

T-9	*T-10*	*T-11*	*T-12A*	*T-12B*	*T-12C*	*T-13*	*T-14*	*T-15*
1.00 (1)	1.00 (1)				1.00 (1)	0.82 (1)	1.00 (1)	
	0.95 (1)		1.00 (1)					
1.00 (1)	1.00 (1)	0.85 (1)					0.92 (1)	1.00 (1)
		0.88 (1)		1.00 (1)				
3.05 (3)	1.48 (1)	1.95 (2)	1.08 (1)		1.04 (1)		1.01 (1)	
	0.96 (1)					0.81 (1)		
1.00 (1)	1.89 (2)							
	2.07 (2)	1.09 (1)				3.32 (4)		
		1.00 (1)				1.09 (1)		
2.17 (2)	1.07 (1)		1.11 (1)		1.30 (1)		1.06 (1)	
2.23 (2)				1.38 (1)		2.31 (2)	3.85 (4)	
0.83 (1)		0.84 (1)	1.66 (2)	1.00 (1)			1.80** (3)	
						+ (1)		
3.89 (4)	1.92 (2)	1.00 (1)	2.33 (3)	0.72 (1)			1.00 (1)	
						1.00 (1)		0.95 (1)
0.88 (1)	1.01 (1)	0.93 (1)			1.10 (1)	0.90 (1)		

TABLE X

THE AMINO ACID COMPOSITION OF SELECTED PEPTIDES ISOLATED FROM TRYPTIC DIGESTS OF TWO δ CHAIN VARIANTS AND ONE γ CHAIN VARIANT

The data are given in residues/mole peptide. The numbers between parentheses refer to the number of amino acid residues expected for the appropriate peptide of the normal δ chain or γ chain. The presence of a tryptophan residue is indicated by a + sign.

	A_2'		*A_2 Flatbush*		*F-Malta-I*		
	T-2	*(T-2)*	*T-3*	*(T-3)*	*T-13A*	*T-13B*	*(T-13)*
Lysine	0.06	(1)			*	1.00	(1)
Histidine							(1)
Arginine	1.00		0.98	(1)			
Aspartic acid	1.05	(1)	1.41	(2)			(1)
Threonine	1.01	(1)					(1)
Glutamic acid			1.86	(1)			
Glycine	0.13	(1)	3.14	(3)		1.20	(2)
Alanine	2.43	(2)	0.60	(2)			(1)
Valine	1.23	(1)	2.98	(3)			(3)
Leucine	1.04	(1)	1.00	(1)			(4)
Phenylalanine						0.86	(1)
Tryptophan	+	(1)					

* Not observed in sufficient amount for an accurate analysis.

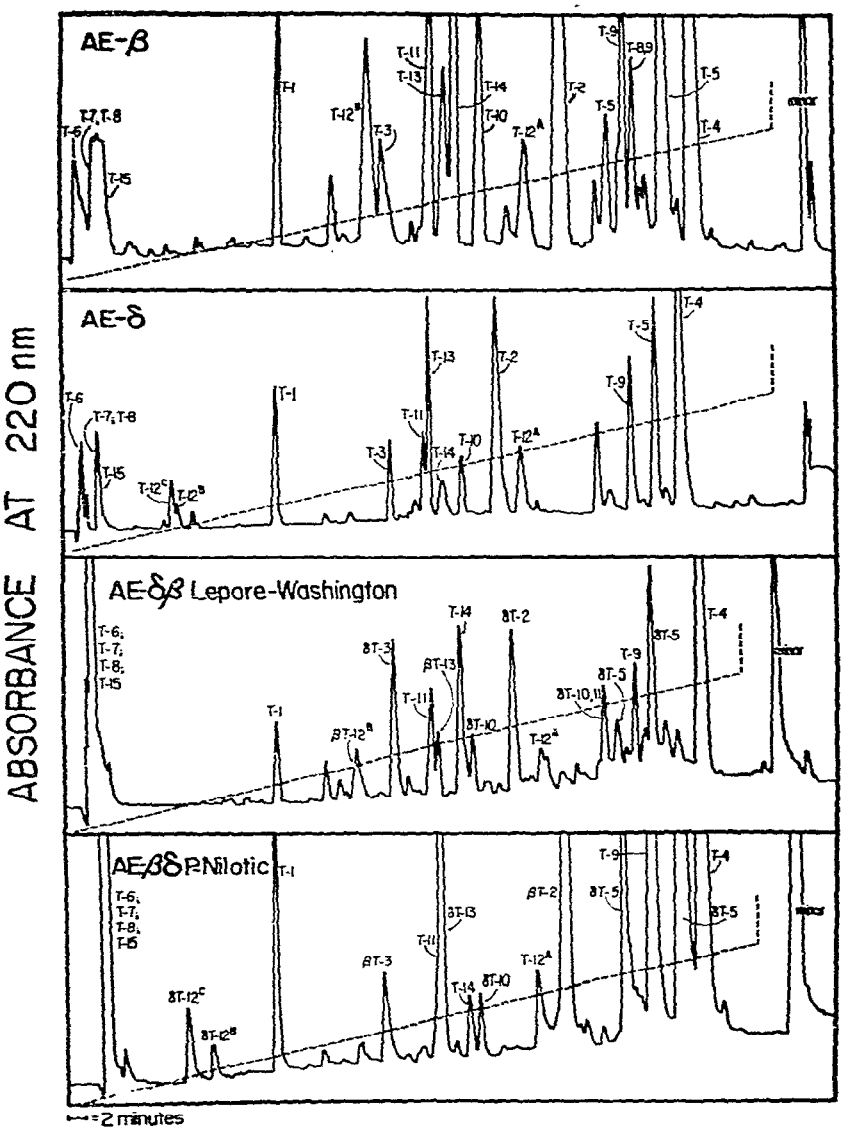

Fig. 5. A comparison of the HPLC of peptides from tryptic digests of aminoethylated β and δ chains and of the aminoethylated hybrid chains of Hb Lepore-Washington and Hb P-Nilotic. Peptides characteristic for either β or δ chains are indicated by an appropriate prefix while identical δ and β peptides lack such an identification.

4,5 in Hb Austin; βT-12^{B},13 in Hb Riyadh). The same is the case for normally occurring double peptides such as αT-1,2; αT-10,11; αT-8,9; βT-8,9. Smaller peptides present in digests of abnormal chains because of the introduction of a lysyl or arginyl residue are eluted earlier than the original peptides. The substitution of an acidic residue (Glu or Asp) for a neutral residue causes the peptide to be eluted later (an exception is the abnormal βT-3 peptide of Hb Connecticut) while the replacement of a neutral residue for an acidic residue reverses the elution pattern. Sometimes a substitution results in a surprisingly large change in the elution of that

peptide which remains unexplained (an example is the abnormal βT-2 of Hb Saki with a Leu → Pro substitution).

The newly developed HPLC procedure has considerable advantages over existing methodology for peptide separation. The method is fast, reproducible and dependable, and only small amounts of material are required. Most peptides eluted from these columns are relatively pure and uncontaminated. Even small changes in the elution profile can often readily be observed and are frequently indicative of a specific peptide being abnormal in structure, which of course has to be confirmed by amino acid and/or sequence analysis. The procedure should not be considered a method that allows a fast definite identification of numerous hemoglobin variants. It only is an additional, but important, tool used for the characterization of variant proteins.

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NOTE ADDED IN PROOF

Recently Schroeder *et al.*[42] used a similar procedure to separate the tryptic peptides of a human hemoglobin variant, HbE, with a β 26 Glu → Lys substitution. The elution buffers were: 0.01 *M* ammonium acetate (pH 6.07), and 40% acetonitrile and 0.01 *M* ammonium acetate (pH 6.07). Development was made with a linear gradient.

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