CHARACTERIZATION OF HUMAN EMBRYONIC HEMOGLOBINS PORTLAND I ($\zeta_2\gamma_2$) AND PORTLAND II ($\zeta_2\beta_2$)

by

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A THESIS

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DEDICATION

This thesis is dedicated to the millions of infants who are not fortunate like others to live after birth.

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INTRODUCTION

A. Statement of the Problem

Structural characterization of globin polypeptides, detailed analyses of the abnormal hemoglobin variants in terms of structure versus function relationship and extensive clinical investigation of the inherited disorders in hemoglobin expression have provided information that is not available for any other eukaryotic system. For these reasons the human globin gene family is a paradigm for studying differential gene activity during development and the molecular basis of genetic disorder. Recent advances in molecular biology have resulted in globin gene mapping and molecular cloning (1). Precise location of an α and α-like globin gene cluster and β and β-like gene cluster has been established (2,3). Previously unidentified globin like "pseudogenes" have also been identified by these techniques (4,5). Also established are the DNA, mRNA and intervening sequences of all the globin genes except for the α-like ζ-globin gene(s). From the standpoint of globin polypeptides the amino acid sequences of all but the ζ-globin chain(s) have been determined (5). The sequences derived from the DNA and/or mRNA by modern sequencing techniques have confirmed and complemented the earlier work of amino acid sequences of various globin chains. Again, information on the primary structure of ζ chain(s) is incomplete despite the fact that several unique tryptic peptides (designated as ζ-peptides) have been reported (7-9). Attempts have also been made to align ζ-peptides with α-globin chains (10,11).

The limited information on the primary structure of the ζ chain(s) can be attributed to: (a) difficulty in obtaining purified embryonic Hb Portland I ($\zeta_2\gamma_2$) and Hb Gower I ($\zeta_2\varepsilon_2$) which are the only

two ζ subunit containing embryonic hemoglobin identified so far; and (b) lack of a suitable chemical method to separate and isolate the ζ chain(s) from the γ, ε or any other globin chain.

The need for a method to purify the ζ chain(s) prior to establishing its chemical structure has become more important since recently two non-allelic genes on the α-like human gene cluster (chromosome #16) have been identified [designated as ζ_1 (3' gene) and ζ_2 (5' gene)] (12) by using genomic blotting techniques. Whether both of these so-called 'zeta' genes code for separate functional polypeptides remained to be determined. Also unknown is whether the embryonic hemoglobins Portland I and Gower I contain one or more, similar or identical zeta chain(s). In addition to the zeta chain it remains to be established whether Hb Portland contains one, two or more kinds of gamma chains. In other words it remains to be shown whether there is more than one type of Hb Portland I molecule.

The purpose of this thesis, therefore, is to contribute towards our understanding of the primary structure of the zeta chain(s) by establishing the following objectives:

(a) To establish a protocol for purifying Hb Portland from the hemolyzate of neonates with homozygous α-thalassemia.

(b) To develop a chemical method for separating and isolating the ζ-chain(s).

(c) To examine the possibility of two kinds of ζ-chain(s) from the globin obtained from neonates with homozygous α-thalassemia.

(d) To examine the possibility of two or more kinds of Hb Portland by identifying the types of zeta, gamma and other globin chains obtained from the purified samples of Hb Portland.

(e) To provide more information about the tryptic peptides and the cyanogen bromide fragments generated from the purified zeta chain(s).

(f) Finally, to provide amino acid sequence or propose the primary structure based on information obtained from the structural studies of the zeta chain(s).

B. Human Embryonic, Fetal and Adult Hemoglobins

The existence of embryonic hemoglobin in man was first described by Huehns (13) in 1961. Two embryonic hemoglobins, Gower 1 (first thought to be ε_4 but later identified as $\zeta_2\varepsilon_2$) and Gower 2 ($\alpha_2\varepsilon_2$) containing embryonic epsilon (ε) chains are found only during the first 10 to 12 weeks of fetal development (13-16). These hemoglobins are synthesized by erythroid cells derived from the yolk sac (17). They are also found in embryos and in normal cord blood (trace amounts). Hb Portland 1 (7,11) is composed of two alpha-like chains (embryonic zeta chains) and two gamma chains. Hemoglobin Portland 1 (henceforth to be called Hb Portland) was first found in a female Chinese infant having multiple congenital anomalies and complex autosomal mosaicism (7) and later reported in infants with hydrops fetalis due to homozygous alpha-thalassemia (18,19). Capp et al. (8) provided the structural evidence that demonstrated a new hemoglobin chain which they designated as the zeta (ζ) chain. Their findings suggested a new hemoglobin chain gene in addition to the then known or postulated globin genes.

Beginning at approximately eight weeks of gestation the embryonic chains are gradually replaced by the adult alpha-globin chain and two different fetal beta-like chains designated as $^{G}\gamma$ and $^{A}\gamma$. The gamma chain differ only by the presence of glycine or alanine at position 136 (20). Therefore, Hb F ($\alpha_2\gamma_2$) or any other gamma chain

containing hemoglobin tetramer can be heterogeneous due to the presence of at least two nonallelic γ globin genes. $^{G}\gamma$ indicates the chain with a glycyl residue at position 136 and $^{A}\gamma$ refers to the chain with an alanyl residue at this position.

Another heterogeneity can exist at position 75 of the $^{A}\gamma$ chain (either isoleucine or threonine). These two chains (21) and the corresponding $^{G}\gamma$ chains are identified as follows (22):

Common Name	Scientific Designation
$A_\gamma I$	γ75(E19)Ile;136(H14)Ala
$A_\gamma T$	γ75(E19)Thr;136(H14)Ala
$G_\gamma I$	γ75(E19)Ile;136(H14)Gly

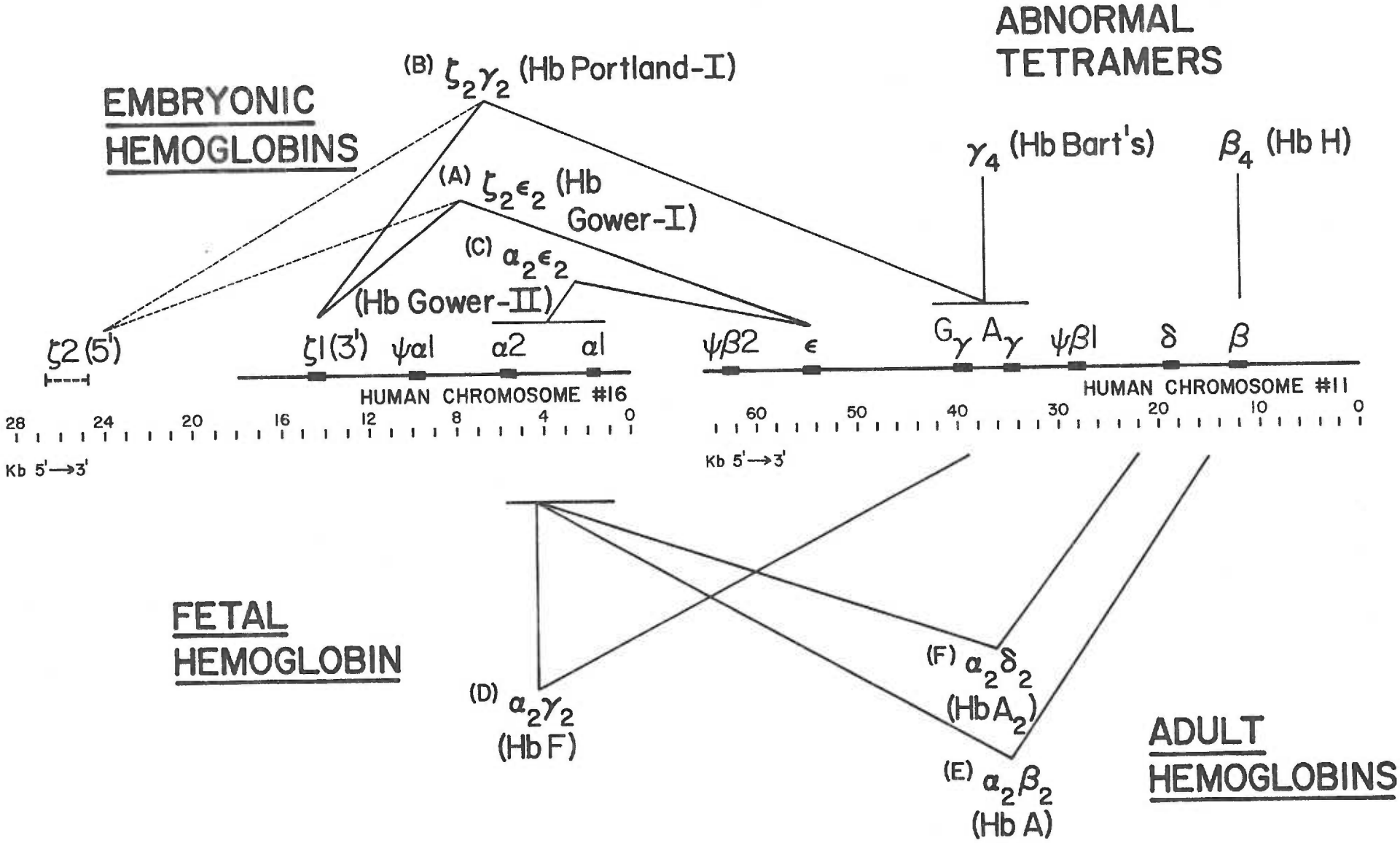
Beginning just prior to birth the gamma-globin chains are gradually replaced by adult beta and delta globin polypeptides. A schematic representation of the precise location of human globin genes, their gene products, the globin chains and the tetrameric hemoglobins they form sequentially during human development is outlined in Figure 1.

The site of erythropoiesis changes from the yolk sac in the early embryo, to the developing liver, spleen and bone marrow in the fetus and finally to the bone marrow in adults (5).

C. Genetic Disorders in Globin Gene Expression

At present more than 350 hemoglobin variants have been described in humans (6). Of these only a small proportion of the variants result in clinically serious defects; the rest of the mutations are silent or harmless. Mutations also occur which are characterized by a reduced level of globin chain production (globin chain $^{+}$-thalassemia)

Figure 1. Schematic representation of the precise location of alpha-like globin genes on the human chromosome #16 (2) and beta-like globin gene cluster on human chromosome #11 (3). Their gene products; the globin chains form tetrameric hemoglobin molecules which are shown sequentially (A-F) during human development. The pseudogenes ($\psi\alpha 1$, $\psi\beta 1$ and $\psi\beta 2$) are believed not to form any functional polypeptides (4,5).


ABNORMAL TETRAMERS
EMBRYONIC HEMOGLOBINS
(B) $\zeta_2\gamma_2$ (Hb Portland-I)
(A) $\zeta_2\epsilon_2$ (Hb Gower-I)
(C) $\alpha_2\epsilon_2$ (Hb Gower-II)
γ_4 (Hb Bart's)
β_4 (Hb H)
ζ2 (5')
ζ1 (3') ψα1 α2 α1
HUMAN CHROMOSOME #16
28 24 20 16 12 8 4 0
Kb 5'→3'
ψβ2 ε Gγ Aγ ψβ1 δ β
HUMAN CHROMOSOME #11
60 50 40 30 20 10 0
Kb 5'→3'
FETAL HEMOGLOBIN
(D) $\alpha_2\gamma_2$ (Hb F)
(F) $\alpha_2\delta_2$ (HbA_2)
(E) $\alpha_2\beta_2$ (Hb A)
ADULT HEMOGLOBINS

or by complete absence of the globin chain production (globin o-thalassemia). Maniatis et al. (1) recently summarized in a review article the information currently available regarding the molecular basis of various types of α and β thalassemia.

In general, mutations can affect the production of any of the known human globin chains, but as Hb A ($\alpha_2\beta_2$) is the predominant hemoglobin in postnatal life, the two most important types of thalassemia are the α and β thalassemias, where α- and β-globin chain synthesis, respectively, are impaired (23-25).

Alpha-thalassemia (α-thal.) has been divided into two groups according to their genotypes which are: α-thalassemia-1 or $\alpha^o\alpha^o/\alpha\ \alpha$ and α-thalassemia-2 or $\alpha^o\alpha/\alpha\alpha$. The homozygous state of α-thalassemia-1 is indicated as HYDROPS FETALIS or $\alpha^o\alpha^o/\alpha^o\alpha^o$, whereas the homozygous state of α-thalassemia-2 is indicated as $\alpha^o\alpha/\alpha^o\alpha$. Hemoglobin H disease, a heterozygosity for α-thalassemia-1, as well as thalassemia-2 is indicated as $\alpha^o\alpha^o/\alpha^o\alpha$. Thalassemia occurs throughout the world and constitutes one of the most common hereditary disorders. The alpha- and beta-thalassemias are inherited as autosomal traits. The actual incidence of thalassemia in various populations can be obtained only on the basis of surveys. In Southern Italian, Sicilian and Greek populations approximately ten percent of the individuals are heterozygotes for beta-thalassemia. In Blacks fewer then five percent are affected with alpha- or beta-thalassemia. The high frequency of thalassemia in some populations is probably due to a protection against falciparum malaria.

Alpha-thalassemia occurs predominantly in people of Asian, Mediterranean and African origin. The severe homozygous form, hydrops

fetalis, occurs almost exclusively in southeast Asia and has been well characterized in Chinese, Thai, Fillipinos, Indonesian, Malaysian, Vietnamese and Cambodians (16,17,26).

In severe homozygous alpha-thalassemia there is a total absence of alpha-chain synthesis (27,28). This homozygous condition is invariably lethal; the affected fetus dies either during the third trimester of pregnancy or, if born alive, within hours or at most one or two days after birth. The fetuses are hydropic with marked edema and ascites (Figure 2). The changes can sometimes be detectable in-utero in midtrimester by means of ultrasound (29). Hb Bart's is the major hemoglobin component together with varying amounts (5-30%) of Hb Portland (18,19) which has a normal oxygen dissociation curve (30) and is believed to help maintain the fetus to term. Hb Portland was originally reported by Capp et al. (7) in a female Chinese infant with multiple congenital anomalies. In certain pathologic conditions embryonic and fetal chain synthesis persists to a later period. For example in D1 trisomy, Hb Gower 1 and Hb Portland I are detectable at birth (31). In homozygous alpha-thalassemia Hb Portland synthesis persists to birth (18,19). Therefore, Hb Portland can be purified from the hemolyzate obtained from subjects with any of these pathologic conditions.

Capp et al. (7,8) identified ζ globin chain and several unique ζ tryptic peptides. Table 1 summarizes the material and methods and Table 2 shows some of the tryptic peptides that have been reported to date by several investigators. It is very clear from the previous work (Tables 1 and 2) that all the investigators except Capp et al. (7,8) used mixtures of various globin chains. Furthermore the sequences

Figure 2. A stillborn fetus of Malaysian (Chinese) origin showing generalized hydrops fetalis and a large placenta. (Courtesy of Dr. Luan Eng Lie-Injo)

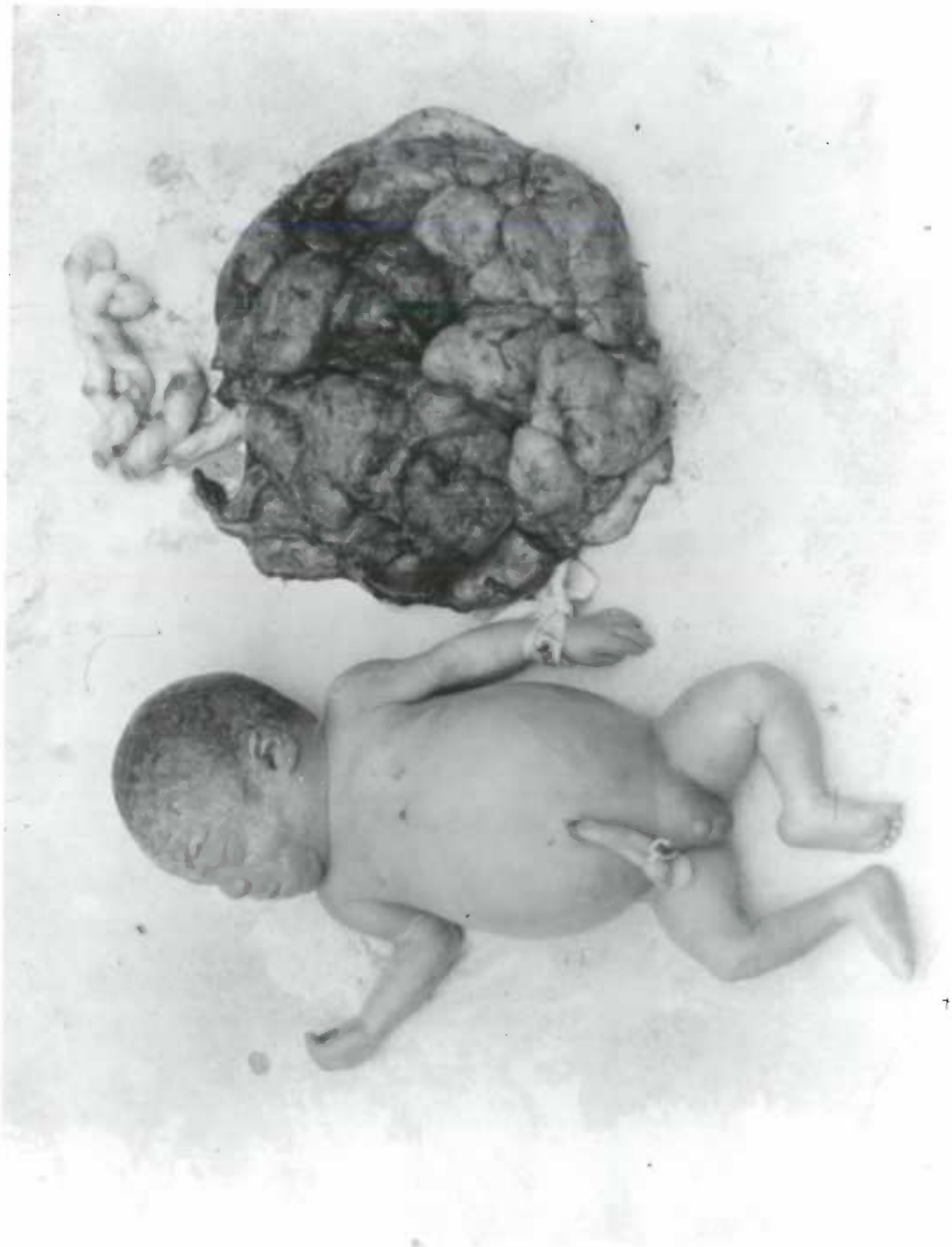


Table 1. Comparison of sample sources and methodologies used by various investigators (7-10,12,32,33) to derive information on the zeta chain. Investigators other than Capp et al. used mixtures of several hemoglobins or globin chains.

TABLE 1

INVESTIGATORS	TYPE OF SAMPLE	METHODS USED TO ISOLATE (a) Hemoglobin	(b) Globin Chain(s)	(c) Tryptic Peptides
Capp et al. (7,8)	Blood from a Chinese female infant with multiple congenital anomalies	Starch gel electrophoresis (Hb Portland 1)	Counter current distribution (AE)	1) A-5 Ion exchange chromatography 2) Rechromatography of each zone by Dowex 50-W 3) Amino acid analysis
Kamuzora et al. (9)	Hemolyzate from a hydropic infant, died immediately after delivery at 32 weeks	Mixture of several hemoglobins	Whole hemolyzate was precipitated to globin and aminoethylated (AE)	1) Fingerprinting of the soluble tryptic peptides 2) Further purification by paper electrophoresis 3) Amino acid analysis
Kamuzora et al. (10)	Blood pooled from 67 human embryos	1) DEAE Sephadex chromatography 2) Paper electrophoresis of the mixture	Mixture of globin chains (non AE)	1) Fingerprinting of tryptic peptides 2) Fingerprinting of peptic peptides of tryptic core
Gale et al. (32)	Material obtained from vacuum abortions of 6-10 weeks gestation	1) Passed through coarse sieve 2) DEAE Sephadex 3) DEAE Cellulose	Mixture of Hb Gower 1 and Hb A (non AE)	1) Tryptic peptide map of mixture 2) Amino acid analysis of various ε and ζ peptides
Rutherford et al.(33)	K-562 human leukemic cells	1) Starch gel electrophoresis of the cells 2) Gel filtration 3) DEAE Chromatography (Hb Gower 1)	Mixture of various globin chains (non AE)	1) Fingerprinting 2) Assignment of peptide based on ninhydrin positive test 3) Amino acid analysis of ε and ζv peptide
Lauer et al. (12)	Genomic human embryonic DNA	Cloned ζ globin gene and the ζ globin gene identified by genomic blotting experiments as ζ1(3') and ζ2(5'), respectively		DNA fragments of ζ1 nucleotide sequence presumably corresponding to ζ73-96 was reported

Table 2. Zeta chain tryptic peptides reported by various investigators. The zeta tryptic peptides have been aligned with α-human globin chain by Kamuzora et al. (10). They based this alignment on amino acid composition of the peptides. The bottom line is based upon DNA sequence work of Lauer et al. (12).

TABLE 2

INVESTIGATORS	ZETA (ζ) PEPTIDES																				
	1-7 (7)	8-11 (4)	12-16 (5)	17-31 (15)	32-40 (9)	41-46 (6)	47-56 (10)	57-61 (5)	62-77 (16)	78-89 (12)	90-92 (3)	93-99 (7)	100-107 (8)	108-114	115-127	128-139	140-141	Possible Extension of Chain	Peptides Which Could not be Aligned	DNA Sequence of ζ1 Fragment
Capp et al. (7,8)		A		B	F			I			G		H	E		C	J	D	1	
Kamuzora et al. (9)			✓		✓		✓	✓	✓	✓	✓				✓		✓			
Kamuzora et al. (10)	1		✓		✓		✓	✓	✓	✓	✓	✓*		✓	✓		✓	D like	4	
Gale et al. (32)				✓	✓			✓			✓	✓					✓			
Rutherford et al. (33)					✓															
Lauer et al. (12)																			ζ1	73-96 DNA sequence

*Obtained from peptic peptide of tryptic core with Arg at ζ99 position (based on alignment with α-chain).

proposed are based on composition of various unique peptides and on assumed alignments with the α chain.

The present work, therefore, is proposed to meet these deficiencies in an attempt to focus on the questions raised in the section of the objectives.

MATERIALS AND METHODS

Blood Samples

Blood from neonates with hydrops fetalis due to homozygous α-thalassemia was obtained from the delivery room of the General Hospital, Kuala Lumpur, Malaysia. The washed red blood cells were packed in dry ice and flown to the United States. Hemolyzates were prepared by the standard method (34,35) using deionized distilled water and toluene. All of these samples were provided through the active collaboration of Luan Eng Lie Injo, M. D., Ph. D., Research Hematologist, University of California Medical School, San Francisco, from neonates designated as CFM, GTT, CKL, YLL, CH and CTY.

Polyacrylamide Gel Electrofocusing

Hemoglobin Portland, Hb Bart's and other fractions were obtained by isoelectric focusing (36) using both analytical and preparative techinques.

Analytical isoelectricfocusing was performed in a 2 mm thick slab of polyacrylamide gel (% T=5, % C=3) containing 2.4% ampholine carrier ampholyte in the pH range 6-8 without glycerol. After a 30 min prerun, 10-20 μl of 1 mg/ml Hb samples predialyzed in 1% ampholine solution were applied to the gel surface on 4x8 mm paper and the separation was continued for 12 hr at 4°C in an LKB 2117 Multiphor Cell, using 10 w from a constant wattage power supply (LKB 2103). Measurement of the pH of the gel was performed using an IEF combination surface electrode (Ingold) connected to digital pH meter (Model 801 S/P) before staining the polyacrylamide gel. The hemoglobin staining was performed according to LKB Notes #250 using bromophenol blue stain.

Preparative Flat Bed Electrofocusing in Granulated Gel

Most technical aspects and the preparation of the Sephadex G-75 Superfine are described in LKB Application Note #198. Five grams of ultrodex (LKB), 100 ml of distilled water, and 5 ml of 6-8 pH range Ampholine (LKB) were gently stirred. The bubble formation in the gel suspension was avoided by performing this procedure in the cold room. The homogeneous slurry was then poured onto the LKB glass plate which already contained three layers of filter paper strips at each end of the tray. The filter paper strips were 10.5 cm long and had been soaked in 5% ampholyte solution (pH 6-8). The slurry was evaporated using a small fan about 70 cm above the tray until the water loss was 37% of the initial slurry weight as determined by weighing the tray on a well-leveled balance.

Prefocusing of the gel was performed at an initial power output of 0.7 watts for 6 hr without the sample. Two ml of the whole hemolysate containing 250 mg of hemoglobin (predialyzed against 1% ampholine solution) was applied near the anode using the LKB sample applicator. The final power was 8 watts constant power at 4°C and the sample was focused in about 12 hr. The separated zones were collected by sectioning the gel bed with the fractioning grid. The hemoglobin components from the focused zones were eluted by transferring the gel sections to the 10 ml disposable column (LKB) using isotonic phosphate buffer (pH 7.4) as eluent. The hemoglobin solution was concentrated and Ampholine was removed in a single step by ultrafiltration using 25-43 mm diameter membranes (Diaflo) at 12 lbs pressure of N_2 in a cold room (4°C). The absorption spectra of the hemoglobin components were obtained by measuring absorption between 480-660 nm on a Beckman DB-GT

spectrophotometer. Globin was prepared by acid acetone precipitation (35) and in some cases it was aminoethylated according to the procedure described later.

Countercurrent Distribution

The distribution apparatus (EC 502 countercurrent fractionator) used consisted of 20 tubes with 10 ml capacity in each tube. The solvent system consisted of sec. butanol, 0.5 M acetic acid and 10% dichloroacetic acid in a volume ratio of 9:10:1 (37). The sec. butanol was purified by fractional distillation until its A_{280}/1cm was less than 0.10. Dichloroacetic acid was distilled before use. The globin solution containing 40-60 mg of aminoethylated globin (38) was dialyzed against the aqueous portions of the system in membrane tubing 3500 mol. wt. cutoff (Spectrator). It was finally concentrated to 10 ml against the aqueous phase before introducing it into the countercurrent apparatus.

Ten ml of dialyzed sample was added to tube #1. The remaining 19 tubes were filled with 10 ml of aqueous solution (lower phase solvent). Ten ml of upper phase solvent (Butanol phase) was then added to tube #1 followed by 20 times rocking and then position changes as shown in the manual (EC Apparatus Corp., Philadelphia). The following scheme for equilibrium time was observed:

Tube #	Equilibrium Time
1	30 min
2	25 min
3	20 min
4-20	15 min

The addition of 10 ml of butanol phase was subsequently made each time

to the above mentioned scheme until 20 transfers were completed.

Recovery of Separated Chain

The aqueous and butanol phases of each tube were each extracted once with an equal volume of cyclohexane. Each cyclohexane layer was extracted two times with 0.1 its volume of water. The aqueous layer and the water washing were evaporated to 0.1 their original volume by rotary evaporation at 45°C. The concentrated solutions were dissolved in 1 ml of 0.1 M acetic acid and then dialyzed against three 250 ml changes of 0.1 M acetic acid for 36 hr using tubing with 3500 mol. wt. cutoff size and finally lyophilized.

Starch Gel Electrophoresis

Hemoglobin Portland, Hb Bart's and other components were obtained by starch gel electrophoresis (39). Starch gel electrophoresis was carried out in Tris-EDTA-boric acid buffer, pH 8.8 and the hemoglobin components were recovered according to the method described by Lie Injo (40).

Cellulose Acetate Electrophoresis

Cellulose acetate electrophoresis was performed using Tris-EDTA-borate buffer at pH 8.8 (41). This method was used for analytical purposes only.

SDS Polyacrylamide Gel Electrophoresis

The system used for electrophoresis was that of Laemmli (42) and O'Farrell (43) with slight modifications as described by Lie Injo (44).

Gel

Polyacrylamide gel (20%) was mixed with 3.8 ml 10% glycerol and 11.2 ml Tris buffer (containing 1.5M Tris and 0.4% SDS), pH 8.8.

Just prior to preparation of the gel, 0.1 ml of 10% ammonium persulfate and 10 µl of N,N,N',N'-tetraethylenediamine (TEMED) are added.

Sample Preparation

A sample of 10 µl of a 0.3-0.5 g% Hb (10-15 g% Hb solution diluted 30 times) or 10 µl of 3-5 mg/ml globin solution in water was mixed with 0.2 ml of a buffer containing 0.1 ml mercaptoethanol, 0.2 ml of 0.5 M sodium phosphate pH 7.0 and 8.7 ml distilled water. Subsequently 20 µl of 10% SDS is added (when preparing globin it is important to add SDS last so that globin does not precipitate). The mixture is heated for 2 min in a test tube placed in a beaker of boiling water. For each run 25 µl of this sample mixture was loaded onto the gel after mixing with 2 µl bromophenol blue, 1 µl mercaptoethanol and 5 µl of 5% glycerol.

After a prerun of 1-2 hr the samples were loaded. At the start of the run 5 v/cm are applied and increased to 35 v/cm after 1 hr. A good separation of globin chains can be achieved after about 9 hr of electrophoresis in the 20% gel.

After the separation the gel was fixed in 12% trichloroacetic acid in water for 1 hr and stained in 0.1% Coomassie blue in methanol-acetic acid solution (0.33 g Coomassie brilliant blue, 150 ml methanol, 150 ml water and 30 ml glacial acetic acid) for one hr. After staining the gel was washed repeatedly in a solution of methanol-water-glacial acetic acid in the proportions 10:10:1. Once destained, the gel was finally soaked in water for several hours and then dried by placing in a Bio Rad gel slab dryer (Model 224) for 1 hr at 45°C.

Polyacrylamide Gel Electrophoresis

Globin chains were separated by the method of Alter et al.

(45) using electrophoresis on 12% polyacrylamide gels, containing 6M urea and 2% Triton X-100, in 5% acetic acid.

Slab gels (12x16 cm x 1.5 mm) with 6 or 12 spaces were employed for analytical electrophoresis using 5% acetic acid as electrophoresis buffer. The sample buffer consisted of 5 ml of deionized 8 M urea, 0.5 ml of glacial acetic acid, 0.5 ml of 2-mercaptoethanol, and 2 mg of pyronin Y. Five-ten μg of globin was mixed with 20 μl of sample buffer. Electrophoresis was performed for 17 hr at 8.5 mA, run at constant current at room temperature.

The gels were stained for 30 min in 0.5% Coomassie brilliant blue, in 7% acetic acid, 30% methanol and destained in the acetic acid-methanol by diffusion. The gels were dried as described above.

NP-40, 8 M Urea Polyacrylamide Isoelectric Focusing

Lyophilized globin samples of 50-200 μg were dissolved in 8 M urea, 3% Non idet P-40 (NP-40), 10% 2-mercaptoethanol, at a concentration of 5 mg/ml, focused in 6% acrylamide slab gels (12.5x2.6 cm x 1.5 mm) containing 8 M deionized urea, 2% pH 6-8 and 0.2% pH 3.5-10 Ampholine (LKB), 3% NP-40 and stained with Coomassie brilliant blue G-250 (46). The gels were prefocused for 2 hr at 200 v current and finally focused for 2 hr 30 min after the application of globin samples.

Cyanogen Bromide Cleavage

Reduction of metsulfoxide prior to CNBr was achieved according to the method of Weihing and Korn (47) with the modification that small amounts of globin (less than 2 mg) were desalted by dialysis (3500 mol. wt. cutoff Spectrator membrane) against 1% acetic acid containing 0.1% 2-mercaptoethanol. The desalted sample was finally lyophilized. Dried sample plus an equal weight of cyanogen bromide were dissolved in 70%

formic acid. The reaction was carried out in the dark at room temperature for 18 hr. This was diluted to 20-fold with water, freeze dried and applied either to a Sephadex G-50 column (48) or Synchropak reverse phase HPLC column for the separation of the cyanogen bromide fragments (49).

Tryptic Peptides from Globin Chains

Tryptic peptide patterns of aminoethylated and non-aminoethylated globin of Hb Portland, Hb Bart's, γ and ζ chains were obtained according to the procedure of Jones (50) and by HPLC procedures recently described by Schroeder et al. (51). Aminoethylation of the cysteine group was achieved according to the method of Jones (38).

A-5 Cation-exchange Chromatography

Two types of Aminex A-5 (Bio Rad) cation exchange peptide chromatography procedures were adopted.

1. Analytical A-5 Chromatography

The aminoethylated and non-aminoethylated tryptic peptides from 0.5-2 mg of hydrolyzate were separated on a 0.9 x 15 cm column of A-5 ion exchange resin using a linear gradient of two pyridine acetate buffers (125 ml each) from pH 3.1 to 5.0 at a flow rate of 30 ml/hr. Of the eluate, 24 ml/hr was diverted to a stream which contained ninhydrin and sodium citrate for development of color, which was recorded at 570 nm.

2. Preparative A-5 Peptide Chromatography

The tryptic peptides of nonaminoethylated samples of 40 mg of Hb Portland (obtained from B #3 of preparative isoelectric focusing of CFM hydrops) were separated on a 0.9 x 32 cm A-5 resin using a linear gradient of 375 ml pH 3.1 pyridine-acetate and 375 ml (275 ml pH 5.1 +

100 ml pH 3.1) pyridine-acetate. The zones from A-5 chromatography were purified by rechromatography on a 0.9 x 16 cm column of Aminex 50X-X4 (Bio Rad) with a linear gradient of 250 ml each of pH 3.1 and 5 pyridine-acetate buffers.

Acid Hydrolysis

Acid hydrolysis was done in 6N HCl containing 0.009% (w/v) phenol in evacuated, sealed ampoules by heating at 110°C for 22 hr unless stated otherwise. Amino acid analyses were made according to the procedures of Spackman, Stein and Moore (52) with a Spinco Model 120 amino acid analyzer equipped with 20 mm light path flow cells (53). Aminex A-5 resin (Bio Rad) was used in the short column for basic amino acids and A-6 in the long column for neutral or acidic amino acids. A 12 cm rather than 5 cm A-5 column was used for the basic amino acids when aminoethylcysteine was present in the sample.

Components of the HPLC System

The HPLC system used for peptide separations consisted of an Altex-Beckman Model 332 gradient liquid chromatograph comprised of a 420 microprocessor system controller, two model 110A single-piston reciprocating pumps, a dynamically stirred gradient mixing chamber, a syringe-loading sample injection valve (Altex Model 210 valve), a stainless steel analytical/preparative column packed with 5 or 10 µl reverse phase material and a Model 100-30 Hitachi variable wavelength detector with an 8 µl analytical flow cell.

The system was attached to the Hewlett Packard's recorder of a HP 5830A gas chromatograph system which was programmed to compute the percent area of the peaks and the retention times. The mobile phase of the effluent was collected in 10x75 mm disposable borosilicate glass

tubes by using an FC-80K micro fractionator (Gilson).

Globin Chain Separations

1. Sample Preparations

(a) Preparation of the Hemoglobin Solutions

Cord blood samples from newborns were collected with EDTA as anticoagulant. The adult samples were obtained from normal laboratory workers. Hemoglobin Portland and Hb Bart's samples were obtained either from starch gel electrophoresis or by preparative G-75 superfine flat-bed isoelectrocfocusing from relatively fresh hemolyzates of neonates with homozygous α-thalassemia. The isolated Hb A and F were concentrated by ultrafiltration under 20 psi in a nitrogen atmosphere. The purity of the samples were tested by starch gel electrophoresis, cellulose acetate electrophoresis or polyacrylamide isoelectricfocusing.

(b) Preparation of the Globin Samples

Various samples of Hb A, Hb F, cord blood hemolyzates, Hb Portland, various components of Hb Bart's and whole hemolyzate of blood from neonates with α-thalassemia were used. After deheming in acid acetone (35) and washing with acetone, the globin was dissolved and dialyzed against water and lyophilized.

The amount of sample for HPLC was 100-250 μg of globin per 250 μl of 49 mM phosphate solution (pH 2.86) for analytical studies or 1 mg to 10 mg (made at a concentration of 1 mg/2 ml of phosphate solution) for preparative studies. In each case the sample was stirred at room temperature for 1 to 2 hr. The sample was centrifuged at 5,000 rpm for 10 min prior to introducing into HPLC system. The sample was injected into a 250 μl sample loop (Altex part #235400) using 250 μl Hamilton syringe for analytical studies; a 2 ml sample loop (Altex part #235401)

was used for preparative purposes.

(c) Columns and Developer for Globin Separations

The specific chromatographic conditions for HPLC are given in the legend to each figure (4 to 6) and in Table 3. In all cases either Waters μ Bondapak C-18 analytical (3.9 mm x 30 cm) or preparative μ Bondapak C-18 (7.8 mm x 30 cm) column was used. Each of these columns was equipped with a Waters guard column (part #84550) filled with Bondapak C-18 Corasil (part #27248).

The solvents consisted of mixtures of HPLC grade methanol (J. T. Baker Chemical Co., Phillipsburg, NJ) and acetonitrile (U.V. high purity solvent grade, Burdick and Jackson Laboratories, Inc., Muskegon, MI) and a solution of 49 mM KH_2PO_4 and 5.4 mM H_3PO_4 per liter at pH 2.86 (54). Solvent 'A': 38% acetonitrile, 9.5% methanol and 52.5% phosphate solution. Solvent 'B': 50% acetonitrile, 5% methanol and 45% phosphate solution. The phosphate solution was made prior to use with deionized water and was filtered using a 0.45 μm type HA-Millipore membrane.

(d) Gradient Programs for Globin Separation by HPLC Method

HPLC program file #1: Microprocessor program file #1 was the most commonly used gradient program. This program required 55 min duration to run and was used for separating various globin chains from non-aminoethylated globin samples of Hb Portland, Hb Bart's and whole globin made from the hemolyzate obtained from homozygous α-thalassemia neonates. This program is designated as the "short duration program" in Table 3. The times and percent of solvent 'B' for HPLC program file #1 are listed below:

TABLE 3

HPLC CONDITIONS USED FOR SEPARATION OF VARIOUS PEPTIDES

TYPE OF SEPARATION	COLUMNS			PROGRAM			SOLVENTS	
	TYPE	Dimensions mm x cm	Guard Column Material	Altex Micro Processor File No.	Duration (Min)	Flow Rate (ml/min)	A	B
GCSASh	micron Bondapak C-18	3.9 x 30	Corosil C-18	1*	55	1.5	49 mM KH_2PO_4 (2.86)	49 mM KH_2PO_4 (2.86)
GCSAL	"	3.9 x 30	"	6	186	1.5	52.5% + MeOH	45% + MeOH
GCSPSh	"	7.8 x 30	"	1	55	3.5	9.5% + ACN	5% + ACN
GCSPL	"	7.8 x 30	"	5	186	3.5	38%	50%
TpSA/P	Lichrosorb C-18	4.6 x 25	Vydac RP	2**	105	1.0	10 mM NH_4AC (pH 6.0)	40% ACN in 'A'
CbSA/P	Synchropak RP	4.1 x 25	Sychropak RP-S	7	100	0.7	0.1% trifluro-acetic acid	50% n-propanol in 'A'

The abbreviations used for the type of separation are: G = globin, C = chain, A = analytical, P = preparative, Sh = short program, L = long program, Tp = tryptic peptides, Cb = cyanogen bromide fragments.

*Flow rate manually changed to 1.5 ml/min from 3.5 ml/min (as programed in file No. 1).

**In addition to ammonium acetate-acetonitrile, phosphate-acetonitrile program was also used with the following solvents: 'A' = 49 mM KH_2PO_4 (2.86) and 'B' = 60% ACN in 'A' (ACN = acetonitrile).

Time (Minutes)	Value of the Function	Duration (Minutes)
0 - 7	10% B	7
(0.5 - 1.5)	Sample injected	1
7.01-12	10 → 60% B	5
12.01-14	60% B	2
14.01-29	60 → 100% B	15
29.01-46	100% B	17
46.01-51	100 → 10% B	5
51.01-54.5	10% B	3.5
54.51-55	10% B	0.5

(e) Sample Loading Procedure for Preparative Separation of Globin Chains

During the preparative separations when the volume of the globin sample to be loaded on μ Bondapak column (7.8 mm x 30 cm) was in excess of 2 ml then additional 2 ml volumes were each injected by using HPLC program file #9. Each time before starting the program (file #9) the sample was loaded in the 2 ml injection loop and the sample loading handle was moved to the inject position. This resulted in direct transfer of 2 ml of sample into the column when program file #9 was started.

The HPLC program file #9 is as follows:

Time (Minutes)	Value of the Function % B	Duration (Minutes)
0-0.58	10	0.58

At all times, loading of a total sample volume exceeding 10 mg of globin/20 ml of KH_2PO_4 (pH 2.86) was avoided. The last 2 ml portion of the globin sample was injected by starting the desired HPLC program file #1 for separating the globin chains.

(f) Procedure for Cleaning the Micron Bondapak Columns

The solvents used were the same as those for program file #1 (globin chain separation). At the end of each preparative separation

the column was cleaned by purging with 2 ml of KH_2PO_4 (pH 2.86) 5-6 times at an interval of three minutes/injection or until there was no significant signal on the recorder at 220 nm. The flow rate was 3.5 ml/min with pump 'B' at 100%. This procedure dislodged most of the bound globin on the reverse phase column without adversely affecting the resolution of the column. Finally a blank run was made by injecting 2 ml KH_2PO_4 (2.86) and using program file #1 in order to determine if the column was cleaned before starting any new preparative separation.

HPLC Program Files #5 and #6

Both of these files were used for separating globin chains from the hemolysate or globin samples and required 186 min duration (termed as "long duration program" in Table 3).

The procedure for analytical separation for hemolyzate was similar to the procedure described by Huisman et al. (55) with a flow rate of 1.5 ml/min. For preparative separations the flow rate was 3.5 ml/min and the column used was a preparative μ Bondapak C18 (7.88 mm x 30 cm).

The gradient program was the same for HPLC programs 5 and 6. Program file #5 was used for preparative purposes using μ Bondapak C18 column (7.8 mm x 30 cm) with a flow rate of 3.5 ml/min while program file #6 was used for analytical purposes using μ Bondapak C18 column (3.9 mm x 30 cm) with a flow rate of 1.5 ml/min.

The times and % 'B' solvent are as follows:

Time (Minutes)	Value of the Function	Duration (Minutes)
0 - 40	10% B	40
(0.5 - 1.5)	Sample injected	1
40.1-140	10 → 100% B	100
140.1-175	100% B	35
175.1-180	100 → 10% B	5
180.1-185	10% B	5
185.1-186	STOP	1

Separation of Tryptic Peptides by RP HPLC

Preparation of Tryptic Hydrolyzates

Hydrolysis with TPCK-treated trypsin (Worthington) was carried out in a solution containing 1 mg of chain/0.2 ml of deionized water and 10 µg of trypsin, adjusted to pH 8.5 with 5% trimethylamine at 37°C for 2 hr. The solution was then acidified to pH 3 with 1N acetic acid.

The tryptic hydrolyzate was lyophilized to dryness, dissolved in 250 µl of 10 mM ammonium acetate (pH 6.07) and centrifuged before the tryptic peptides were separated by HPL chromatography.

Developer and HPLC Conditions

Ammonium acetate-acetonitrile system (56) was used for the initial separations. Rechromatography of some tryptic peptides was achieved by using a phosphate buffer-acetonitrile system (56). The ammonium acetate was 10 mM (0.77 g/liter) and was brought to pH 6.07 with 5-6 drops of 5N acetic acid. The phosphate buffer was 49 mM KH_2PO_4 (6.66 g/liter) and 5.4 mM H_3PO_4 at pH 2.86. Both of these buffers were freshly prepared, filtered and degassed prior to use. Flow rates of developer were 1 ml/min and the detection was made at 220 nm. After pooling the fractions the water and ammonium acetate were volatized by rotary evaporation or by lyophilization.

Tryptic peptides were separated with an Altex Lichrosorle C-18 5 µm column (4.6 mm x 25 cm) attached to a guard column packed with

Vydac-RP packing material, using the gradient system described by Schroeder et al. (56).

The gradient program used for tryptic peptide separation using either ammonium acetate solvents or potassium phosphate solvents required a total of 106 min. Both analytical and preparative separations were made using HPLC program file #2.

HPLC program file #2 is as follows:

Time (Minutes)	Value of the Function % B	Flow Rate (ml/min)	Duration (Minutes)
0 - 5	0	1.0	5
5.1- 90	0-100	1.0	95
95.1-100	100-0	1.0	5
100.1-105	0	1.0	5
105 - 106	0	0	1

Separation of the Cyanogen Bromide Fragments by RP-HPLC

(a) Preparation of the Sample

After cyanogen bromide cleavage the peptides were recovered by lyophilization and dissolved in 0.1% (v/v, 0.013M) trifluoroacetic acid in water. Protein concentration ranged from 1 to 2 mg/ml.

(b) Developer and HPLC Conditions

Separation of the cyanogen bromide fragments was carried out by reverse phase HPLC according to the conditions recently described by Mahoney and Hermodson (57). The analyses were performed on Synchropak RP (4.1 mm x 25 cm; cat. #CR103-25) attached to a guard column packed with Synchropak RSC (cat. #R-000). The flow rate was maintained at 0.7 ml/min and the separation was carried out at room temperature.

The developers included consisted of a solvent 'A' which was 0.1% trifluoroacetic acid in water and solvent 'B' which was 50% n-propanol in 0.1% trifluoroacetic acid.

(c) HPLC Gradient Program for CNBr Fragments

The HPLC gradient program file #7 was used to separate cyanogen bromide fragments. This program ran for 123 min.

HPLC Program File #7 is as follows:

Time (Minutes)	Value of the Function % B	Flow Rate (ml/min)	Duration (Minutes)
0 - 2	0	0.7	2
2.1-102	0-100	0.7	100
102.1-107	100	0.7	5
107.1-117	100-0	0.7	10
117.1-122	0	0.7	5
122 - 123	0	0	1

RESULTS

I. SEPARATION OF HEMOGLOBIN COMPONENTS OF HYDROPIC HEMOLYSATES

Starch Gel Electrophoresis -- Electrophoresis of whole hemolyzate at pH 8.6 produced four bands. These are designated as components I (major band), II, III and IV (faint band) as shown in Figure 3. Based on tryptic peptide pattern analysis of nonaminoethylated globin by Aminex A-5 cation exchange peptide chromatography (to be referred to as A-5 chromatography), component I had a pattern identical to the γ chain and hence was concluded to be Hb Barts (γ_4). This was further confirmed by separation of $^{G}\gamma$ and $^{A}\gamma$ chains from component I of CKL hydrops and several other neonates by a HPLC method (Figure 4). Amino acid analysis of globin from component I was also identical to the γ chain (Table 4).

The tryptic digests of components II and III when separated by A-5 chromatography were found to contain a unique tryptic peptide designated as zone XVIII. Its composition was that of ζI. In addition, several other differences were found for the tryptic digests of components II and III compared to the γ chain tryptic digest. Hence both of these components were concluded to be ζ containing hemoglobin components. Further examination of component III from CTY hydrops (Figure 6) and CKL hydrops (Figure 5F) by HPLC method also confirmed the presence of ζ chain, very small amounts of $^{G}\gamma$ and $^{A}\gamma$ and another globin chain with a retention time of about 15 minutes. Because the elution position of this chain is identical to that of the β globin chain of Hb A, it is designated as $\beta^{?}$ chain. The designation of $\beta^{?}$ is also based on (a) the resemblance of its tryptic peptide pattern with β globin tryptic peptide pattern and (b) the presence of a tryptic peptide with retention

Figure 3. Separation of various hemoglobin components by starch gel electrophoresis using tris EDTA boric acid buffer pH 8.6. After separation the gel was stained with benzidine and o-dianisidine. (Courtesy of Dr. Luan Eng Lie-Injo)

⊕
⊖
– Hb H (?)
– Hb Bart's (COMP. I)
– Hb PortlandI(COMP. II)
– Hb A
– Hb PortlandII(COMP. III)
– Hb F
– Hb PortlandIII(COMP. IV)
– Hb A2
Normal Adult –
Normal Adult –
Neonate with Homozygous α-thal (Hydrops fetalis) –
Newborn infant with Hb Bart's –

Figure 4. Preparative separation of Hb Bart's (γ_4) globin on Waters μ Bondapak C18 preparative column (7.8 mm x 30 cm). The hemoglobin was obtained from CKL hydrops and separated by starch gel electrophoresis as component 1. The reverse phase was attached with a guard column (containing C18 Corasil packing) and the separation was made at a flow rate of 3.5 ml/min using 55 min duration program (GCSPSh Program) and a 2 ml sample injection valve.

A. Blank run was made by injecting 2 ml of 49 mM KH_2PO_4 (pH 2.86) to be called phosphate solution. The column was cleaned by the procedure given in 'D' below.
B. Separation of $^{G}\gamma$ and $^{A}\gamma$ chains from 1 mg Bart's globin which was dissolved in 2 ml of phosphate solution and stirred for 2 hrs at room temperature prior to sample loading.
C. Immediately after 'B' above a blank run was made by injecting 2 ml of phosphate solution.
D. Column cleaning procedure: While leaving B=100%, the column was purged by six repeated injections of 2 ml phosphate solution at intervals of about 3 min each. After cleaning %B was changed from 100% to 10% in 5 min. The column was equilibrated at 10% B for 10 min with a flow rate of 3.5 ml/min before injecting another sample.

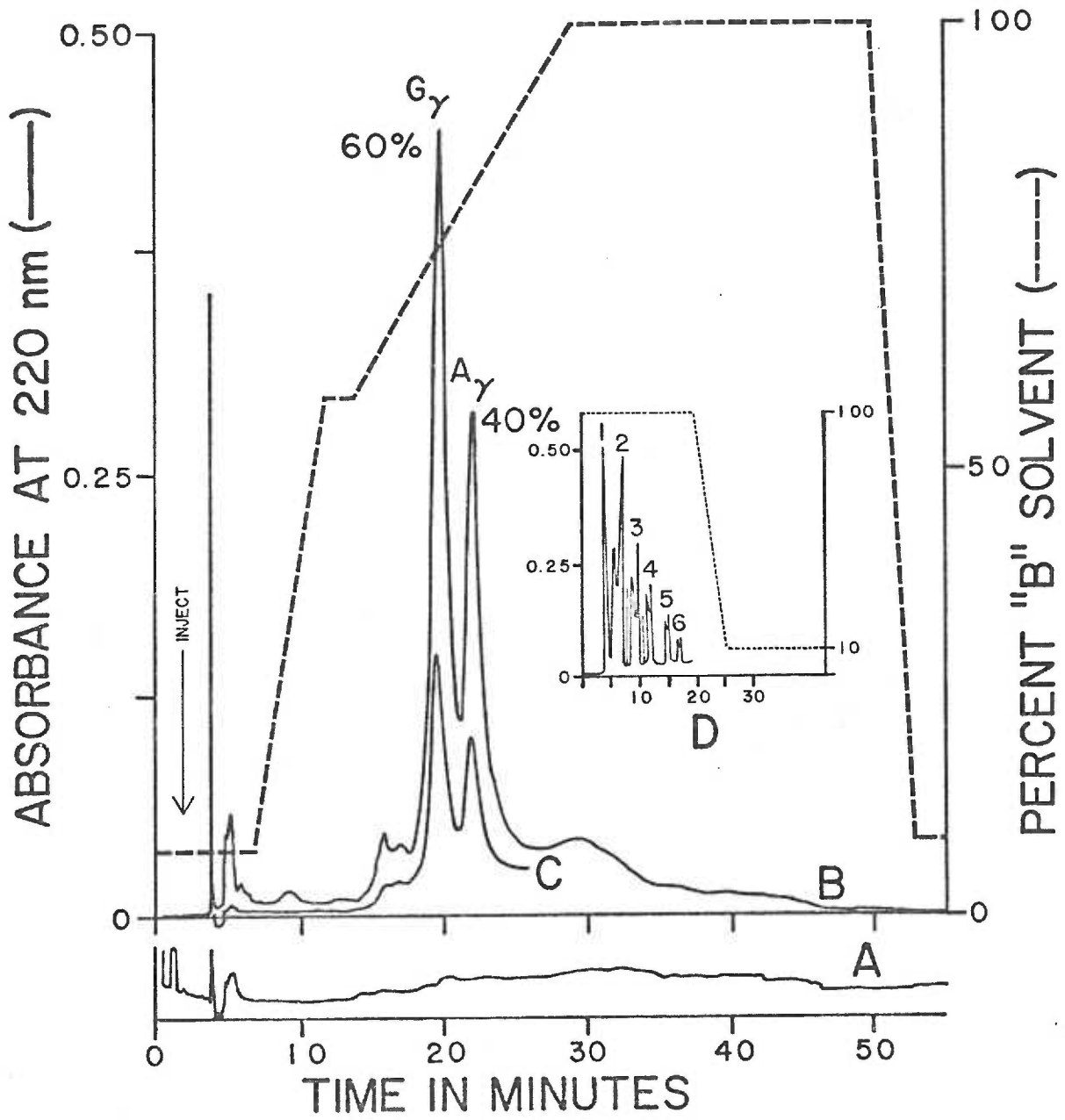

0.50
0.25
0
ABSORBANCE AT 220 nm (——)
100
50
0
PERCENT "B" SOLVENT (- - - -)
G_γ
60%
A_γ
40%
INJECT
C
B
A
2
3
4
5
6
D
10
20
30
40
50
TIME IN MINUTES

TABLE 4

Amino Acid Analyses of Various Purified Globin Chains from Hydropic Blood

Amino Acid	α*	+ζ RT:37 (CKL)	+G_γ RT:19 (CFM)	γ*	β*	+β? RT:15 (CKL)	ε*	δ*
Lysine	11	10	12	12	11	13	14	11
Histidine	10	7	7	7	9	9	7	7
Arginine	3	6	3	3	3	3-4	2	4
Aspartic Acid	12	9	13	13	13	11-12	12	15
Threonine	9	12	10	10	7	8-9	6	5
Serine	11	13	11	11	5	8-9	9	6
Glutamic Acid	5	9	12	12	11	11-12	12	12
Proline	7	5	4	4	7	9	6	6
Glycine	7	7	13	13	13	13-14	9	13
Alanine	21	16	11	11	15	14-15	17	15
Half Cystine	1	(1)	1	1	2	(1)	1	0
Valine	13	9	13	13	18	10-11	13	17
Methionine	2	(1)	2	2	1	(2)	3	2
Isoleucine	0	7	4	4	0	1	4	0
Leucine	18	17	17	17	18	18	17	18
Tyrosine	3	3	2	2	3	3	2	3
Phenylalanine	7	7	8	8	8	7	9	8
Tryptophan	1	(2)	(3)	3	2	(1)	3	2
Total or Assumed Total	141	(141)	(146)	146	146	(146)	146	146

*Values derived from the established sequence.
+Expressed as molar ratios.
( ) Assumed values

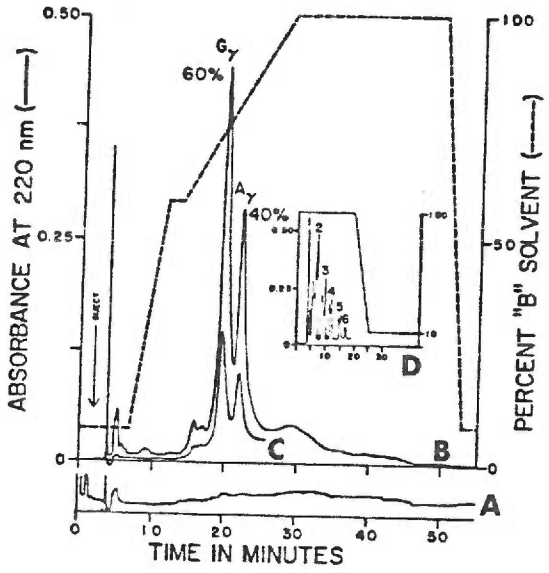
Figure 5. Globin chain separation by HPLC program GCSPSh.

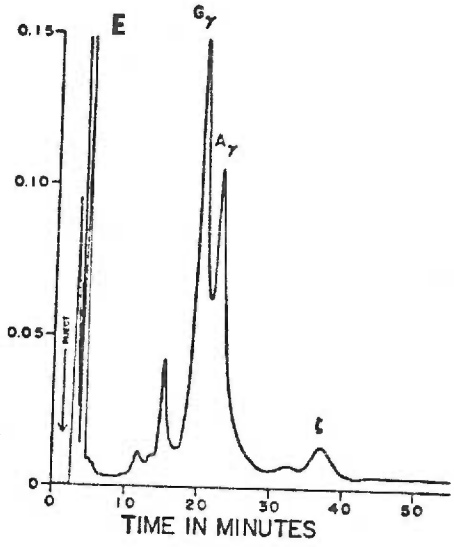
A-D. HPLC procedure for separating 1 mg of Hb Bart's globin from CKL hydrops as shown in Fig. 10.

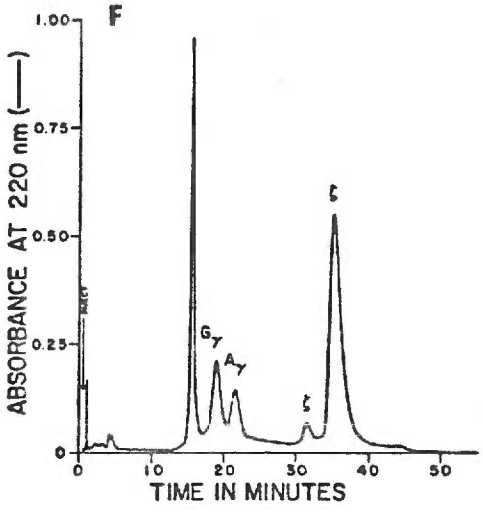
E. About 0.5 mg of whole globin from CKL hydrops.

F. About 6 mg of Hb Portland II globin from CKL hydrops (separated by starch gel electrophoresis).

G. About 5 mg of Hb Portland I globin obtained from GTT hydrops (see text for purification protocol).


ABSORBANCE AT 220 nm (——)
PERCENT "B" SOLVENT (- - -)
TIME IN MINUTES
60%
40%
A
B
C
D


E
TIME IN MINUTES


F
ABSORBANCE AT 220 nm (——)
TIME IN MINUTES

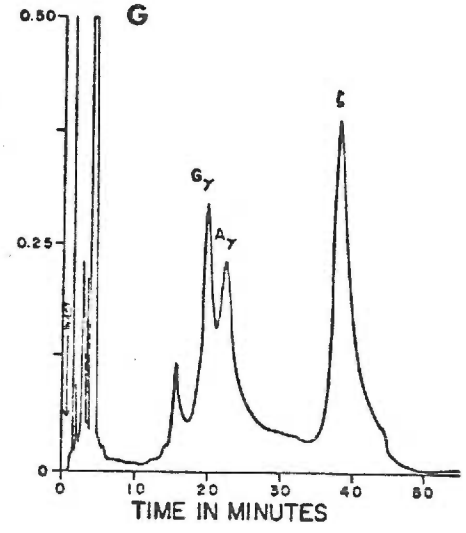
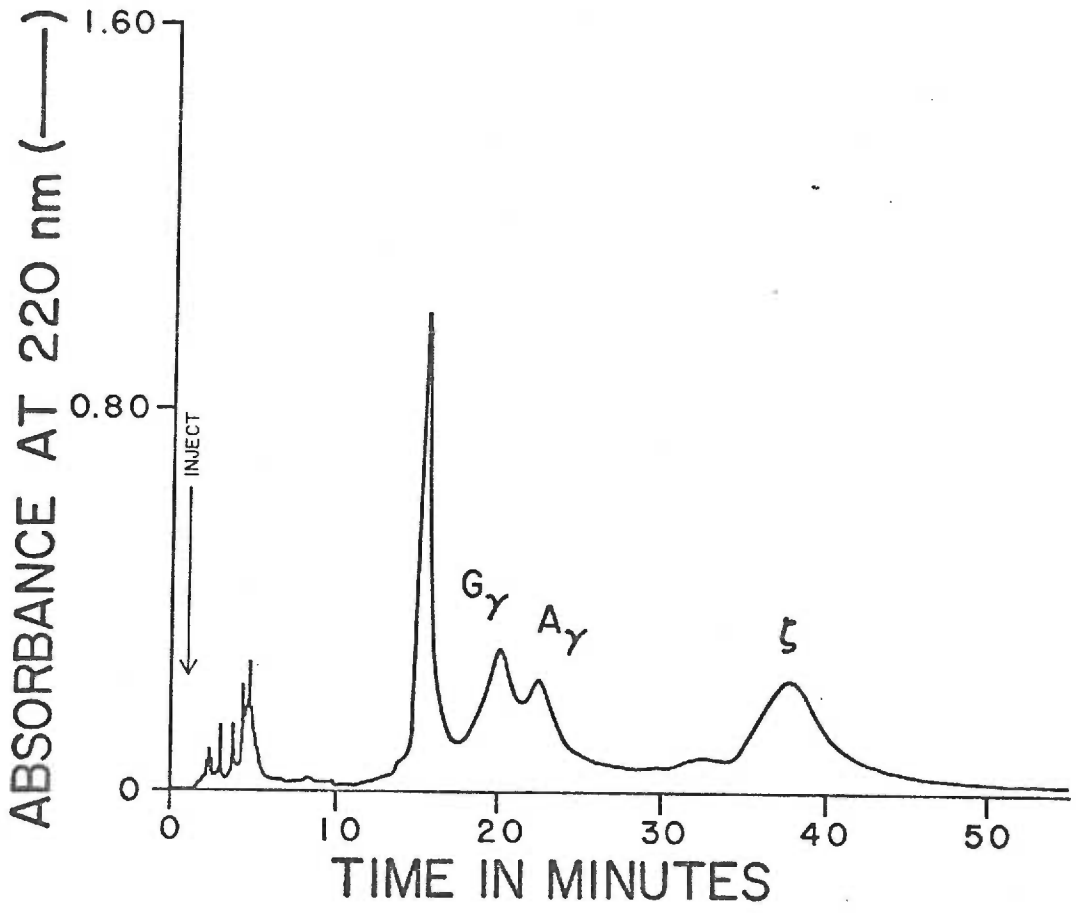

G
TIME IN MINUTES

Figure 6. Separation by HPLC procedure of about 5 mg of Hb Portland II globin obtained from CTY hydrops as component III by starch gel electrophoresis. The HPLC conditions are identical to those described in Fig. 5.


ABSORBANCE AT 220 nm (——)
1.60
0.80
0
INJECT
G_γ
A_γ
ζ
0
10
20
30
40
50
TIME IN MINUTES

time and composition of βT-3. The ζ and $\beta^?$ chains appeared to be present in approximately equal molar amounts in component III. Therefore, component III is believed to have a globin composition of $\zeta_2\beta^?_2$ with a very small amount of $^G\gamma$ and $^A\gamma$ as contaminants either from Hb Bart's or perhaps Hb Portland I ($\zeta_2\gamma_2$). Based on these observations component III is tentatively assigned the designation of Hb Portland II ($\zeta_2\beta^?_2$). The amino acid analyses of HPLC separated RT-15 ($\beta^?$) globin and RT 37 (ζ) globin chains from CKL Hb Portland II is shown in Table 4.

A hemoglobin with the globin composition of Hb Portland I ($\zeta_2\gamma_2$) discovered earlier by Capp et al. (6) was purified from GTT hydrops (Figure 5G) as well as from CFM hydrops (Figure 7) by isoelectric focusing and starch gel electrophoresis. Component II of starch gel electrophoresis is believed to be Hb Portland I, whereas component III is identified as Hb Portland II in at least three hydrops (CKL, CTY and CH).

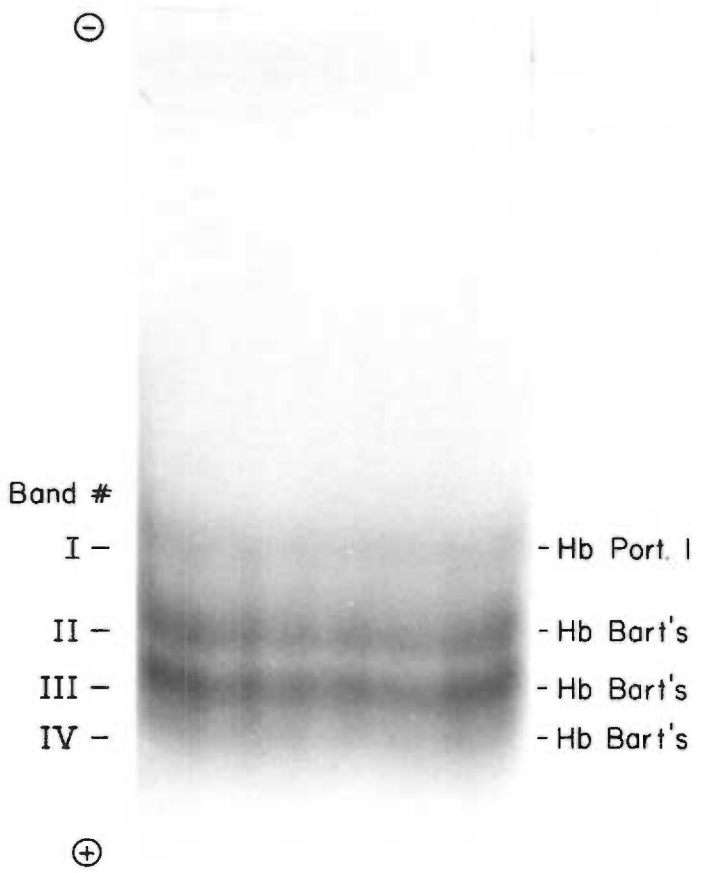
Isoelectric Focusing -- Isoelectric focusing patterns (Figures 8 and 9) of whole blood from the suspected hydropic neonates confirms the absence of Hb A, Hb F, Hb A_2 or any other alpha-chain containing hemoglobin. This demonstrates that the material used in this study came from neonates with hydrops fetalis due to homozygous alpha-thalassemia.

The results of isoelectric focusing of hemolyzates from three different hydropic neonates is as follows:

(i) CFM Hydrops. Figure 10 shows the separation of about 250 mg of whole hemolyzate of CFM hydrops by preparative isoelectric focusing. Sixteen hours (Figure 10A) or eighteen hours (Figure 10B) electrofocusing resulted in the separation of six bands (designated as 1-6). Based on tryptic peptide patterns of nonaminoethylated globin

Figure 7. Further purification of Hb Portland I obtained from band #5 of preparative isoelectricfocusing of CFM hydrops. Hb Portland I (band I) and various Hb Bart's components (bands II to IV) were separated by starch gel electrophoresis. Bands I-IV from starch gel electrophoresis were identified by analytical cellulose acetate electrophoresis (lanes 1-4). Also shown in lanes 5-7 are Hb F, Hb A and Hb A and C, respectively.

STARCH GEL ELECTROPHORESIS
(pH 8.8)



CELLULOSE ACETATE ELECTROPHORESIS
(pH 8.8)

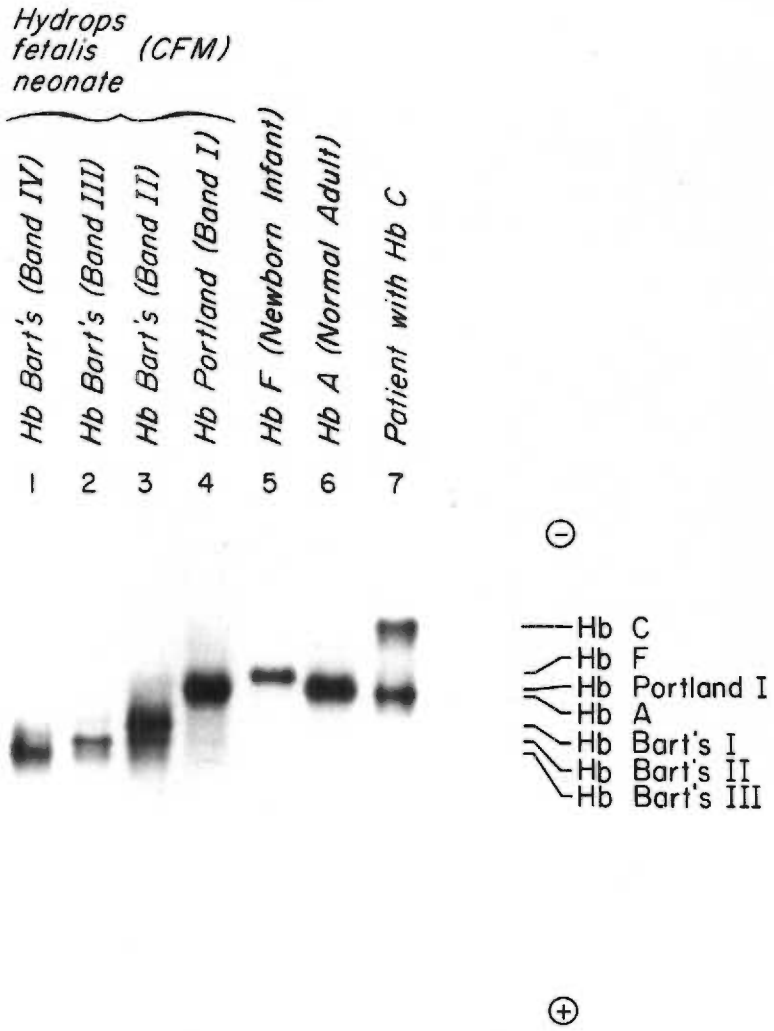


Figure 8. Analytical polyacrylamide gel electrofocusing (pH range 6-8) of hemoglobins from (1) Hb A; (2) cord blood; (3) neonate with hydrops fetalis due to homozygous α-thalassemia; (4) Hb Bart's from band #3 preparative isoelectricfocusing; and (5) purified Hb Portland 1 (Hb Portland was further purified from band #5 preparative isoelectricfocusing by starch gel electrophoresis pH 8.6 according to the procedure described in the text.

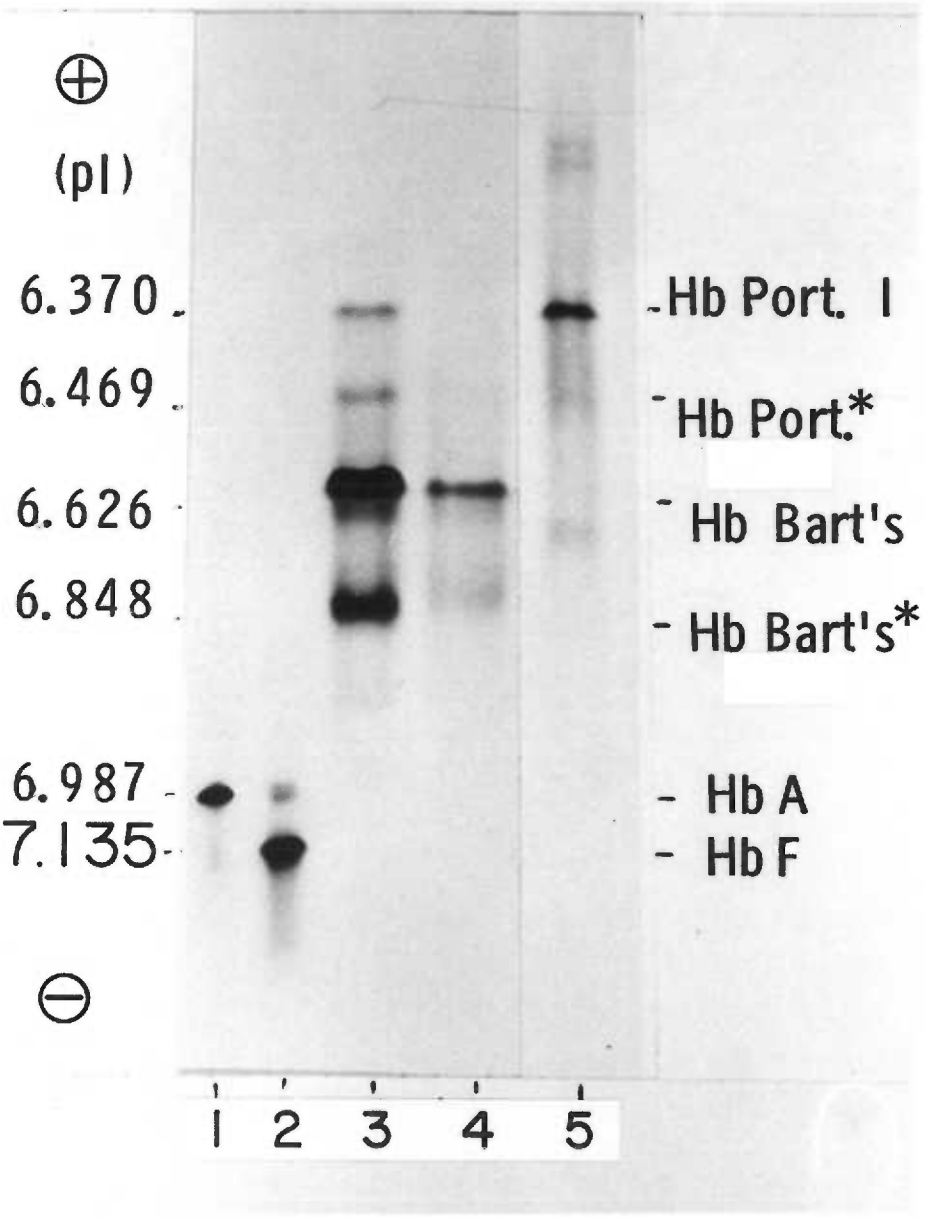

⊕
(pI)
6.370
6.469
6.626
6.848
6.987
7.135
⊖
Hb Port. I
Hb Port.*
Hb Bart's
Hb Bart's*
Hb A
Hb F
1
2
3
4
5

Figure 9. Analytical polyacrylamide isoelectricfocusing of various hemoglobins in 6-8 pH range gel.

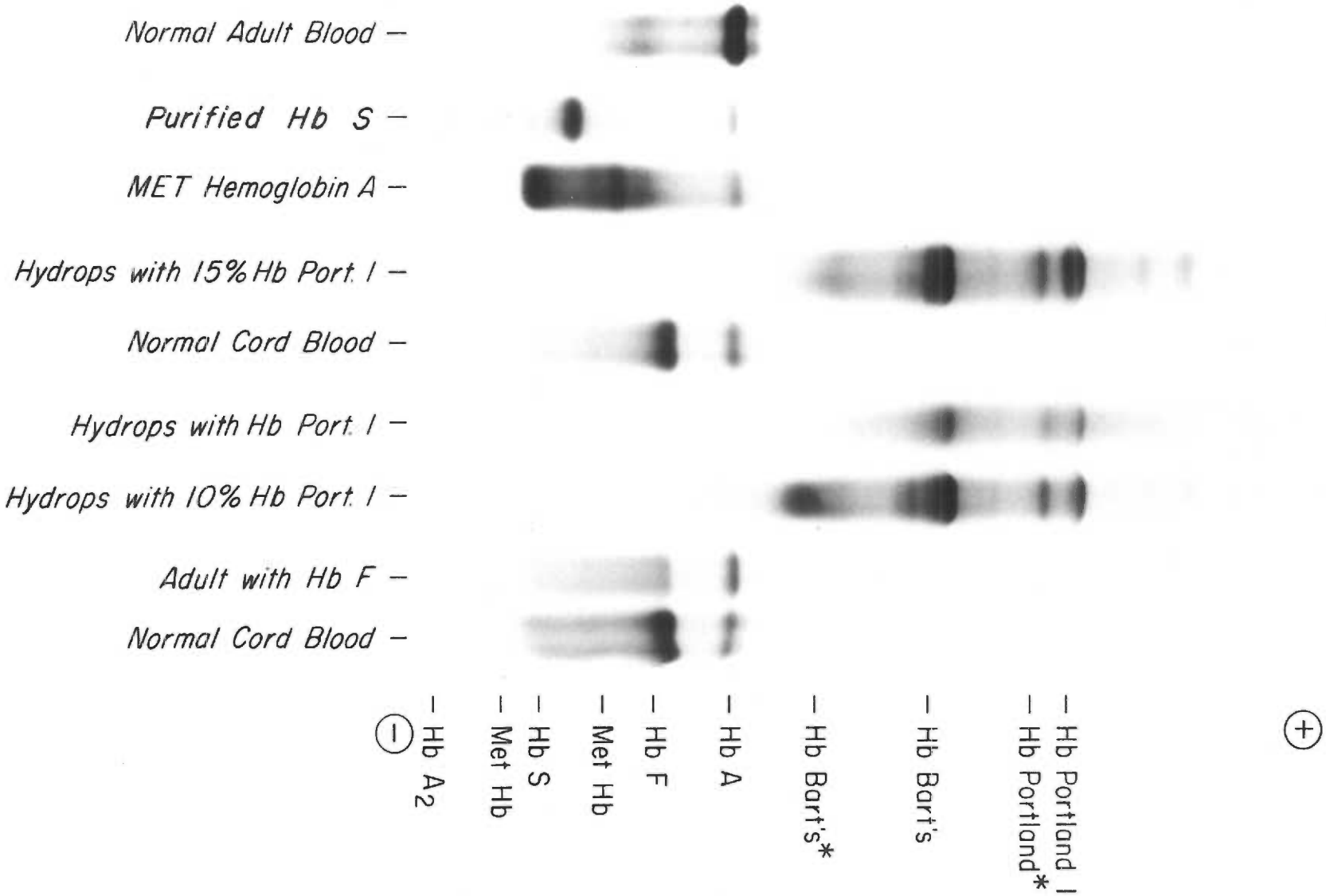

Normal Adult Blood
Purified Hb S
MET Hemoglobin A
Hydrops with 15% Hb Port. I
Normal Cord Blood
Hydrops with Hb Port. I
Hydrops with 10% Hb Port. I
Adult with Hb F
Normal Cord Blood
Hb A2
Met Hb
Hb S
Met Hb
Hb F
Hb A
Hb Bart's*
Hb Bart's
Hb Portland I
Hb Portland*
⊖
⊕

TABLE 5

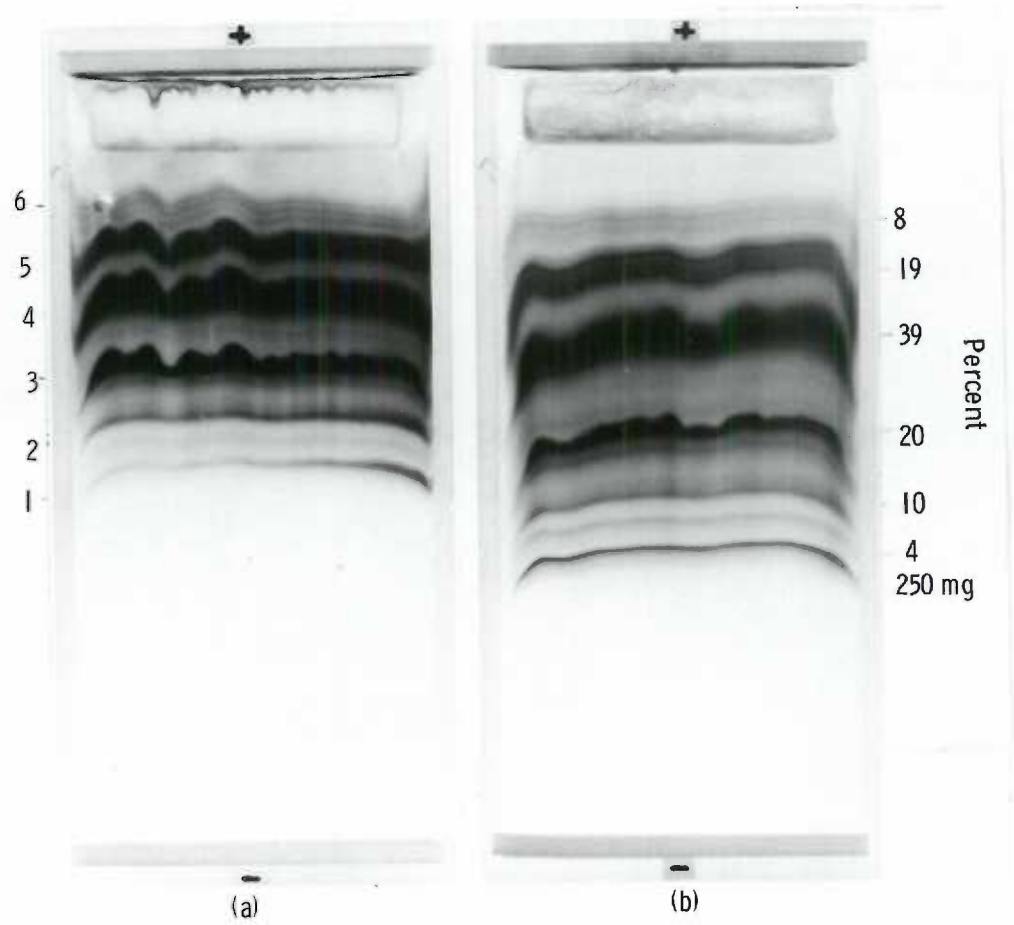
Isoelectric Points of Hemoglobin

Human Hemoglobin	pI (±0.01)	Others		
	Present Work	Drysdale et al. (58)	Baccaria et al. (59)	Krishnamoorthy et al. (60)
Hb H	6.250			
Hb Portland I	6.370			
Hb Portland*	6.469			
Hb Bart's	6.626			
Hb Bart's*	6.848			
Hb A_{1a}	6.860			6.88
Hb A_{1b}	6.872			6.92
Hb A_{1c}	6.978		6.92±0.01	
Hb F_1	6.980	6.90		
Hb A	6.987	6.95	6.97±0.01	6.95
Hb F	7.135	7.15		
Hb A (met)	7.180			
Hb S	7.300	7.25		
Hb A_2	7.437	7.40		7.38

Isoelectric points (pIs) were determined by direct measurement on the surface of gel using IFF Comb. electrode. Each value represents a mean of at least three determinations.

*Based on Aminex 5 peptide chromatography separation pattern of tryptic peptides (see text).

Figure 10. Preparative flatbed electrofocusing of hemolyzate from CFM neonate with hydrops fetalis due to homozygous alpha-thalassemia: (a) shows the result of 250 mg hemoglobin in a 6-8 pH gradient at 6 hr prefocusing + 10 hr sample focusing, and (b) same at 12 hr sample focusing.


+
6
5
4
3
2
1
−
(a)
+
8
19
39
20
10
4
250 mg
Percent
−
(b)

separated by A-5 chromatography, bands 1, 2 and 3 had patterns identical to the gamma-chain pattern. Hence these are designated as Hb Bart's. This was further confirmed by HPLC method of chain separations. Bands 1-3 had similar $^{G}\gamma$ and $^{A}\gamma$ ratios. The tryptic peptides of bands 4 and 5 when separated by A-5 chromatography (Figure 11) were found to contain the unique tryptic peptide zone XVIII (ζI). Hence both of these bands are designated as zeta-containing hemoglobin components. Band 6 was not analyzed in detail but it was found not to contain any significant amount of the zeta chain.

(ii) GTT Hydrops. Figure 12B shows the separation of about 250 mg of whole hemolyzate of GTT hydrops by preparative isoelectric focusing. Electrofocusing of relatively older hemolyzates which could not otherwise be separated by starch gel electrophoresis, perhaps due to aging or partial denaturation of the sample, resulted in bands designated as 1*-4*. The tryptic peptides of nonaminoethylated globin chains of these bands gave patterns by A-5 chromatography as follows: Band 1* was like the gamma chain or Hb Bart's thus corresponds to bands 1-3 of CFM or YLL hydrops. Bands 2* and 3* contain zeta chain as indicated by the presence of the unique XVIII peptide zone and hence correspond to bands 4 and 5, respectively, of CFM or YLL hydrops. Band 4* had a gamma chain pattern and is believed to be Hb Bart's.

Separation of globin chain of whole globin from GTT hydrops by HPLC method (Table 6) failed to show any significant amount of the R.T. 15 or $\beta^{?}$ globin chain. Thus it is concluded that GTT hydrops does not have detectable amounts of Hb Portland II ($\zeta_2\beta^{?}_2$). Hb Portland I from GTT hydrops was purified by starch gel from band 3* (corresponding B #5). The composition of the constituent globin chains and the purity

were confirmed by HPLC separations of about 0.5 mg of hemoglobin using both the long duration, GCSAL program, as shown in Figure 13C as well as the short duration, GCSASh program (Figure 5G).

(iii) YLL Hydrops. Figure 12A shows separation of about 250 mg of whole hemolyzate of YLL hydrops by preparative isoelectric focusing. Hydrops YLL and CFM (Figure 10) were found to have identical pattern of separation by electrofocusing.

Determination of Isoelectric Points -- Figures 8 and 9 show separations of various hemoglobin components by analytical polyacrylamide gel isoelectric focusing using the 6-8 pH range of ampholytes. Isoelectric points (pIs) of these components were measured by direct measurement of pH at the surface of the unstained gel using an isoelectric focusing combination electrode (Ingold). Each value in Table 5 represents a mean of at least three determinations on two different gels. By these measurements the isoelectric point of Hb Portland I was found to be 6.37 and Hb Bart's major band was 6.62 (Figure 8). The same figure also shows bands designated as Hb Portland* at a pI of 6.47 and Hb Bart's* at a pI of 6.84 from CFM hydrops. They were given these designations because the Hb Portland* band on A-5 chromatography gave tryptic peptide patterns which contained the unique XVIII peptide (ζI), whereas Hb Bart's* pattern was identical to that of the gamma chain. Why these bands had pI's different from their main components was not investigated further? The fact that band #5 of CFM hydrops (see Figure 8) contains Hb Portland I with a pI of 6.37 and band #3 contains Hb Bart's (major band) is confirmed from channels 5 and 4 of Figure 8. The purified hemoglobins for channels 5 and 4 were obtained separately after the starch gel electrophoresis of

Figure 11. Peptide pattern of complete tryptic hydrolyzate of 40 mg nonaminoethylated globin obtained from B #5 of preparative isoelectricfocusing (a mixture of Hb Portland I and Hb Bart's) was treated with TPCK trypsin and then applied on 0.9 cm x 30 cm Spinco 15A resin, temperature 50°C. Development was with a linear gradient of 500 ml of pyridine-acetate buffer beginning at pH 3.1, 0.2 M pyridine and ending at pH 5.0, 2.0 M pyridine. The column flow was 30 ml/hr and ninhydrin flow was 15 ml/hr.

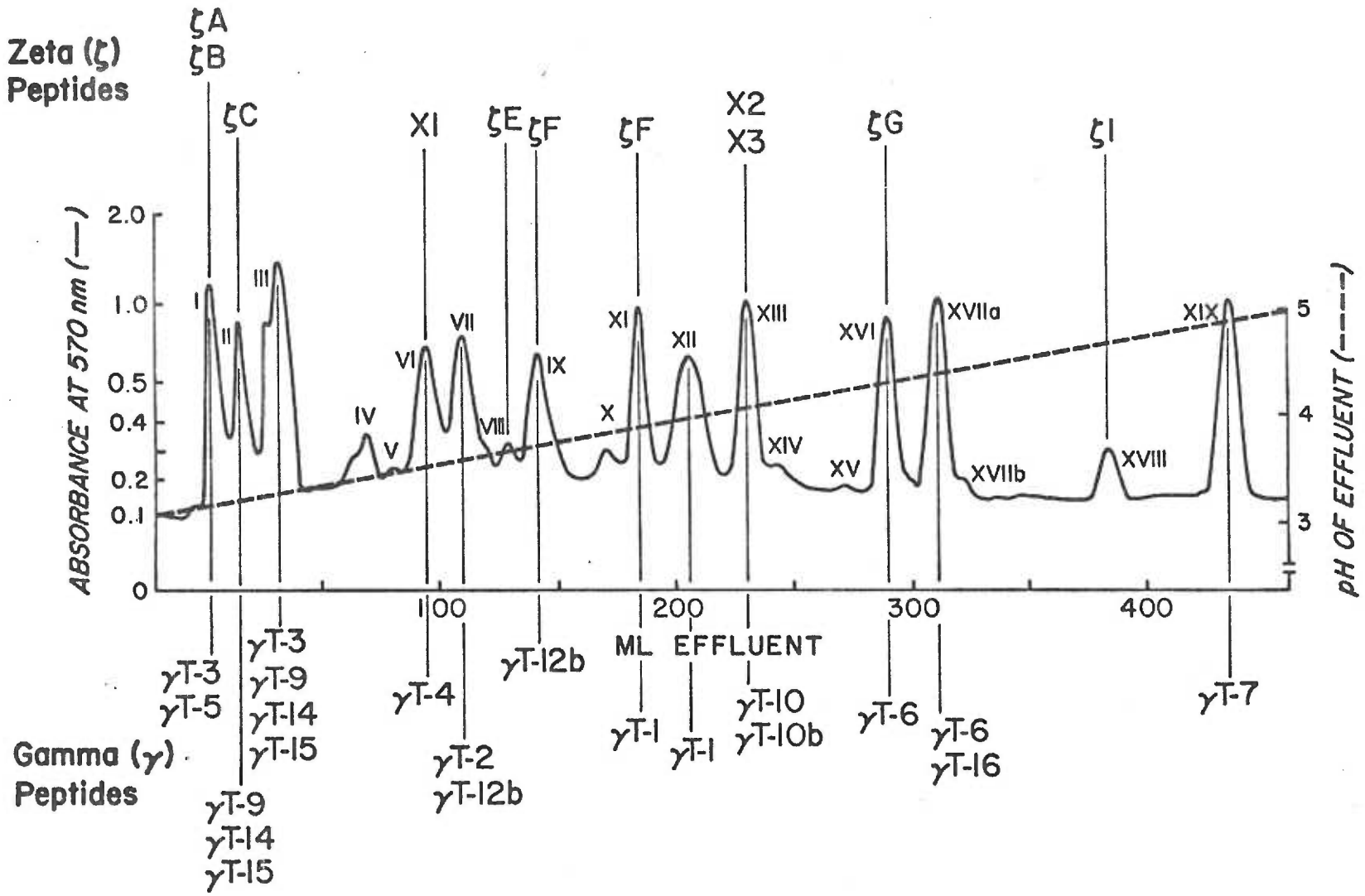
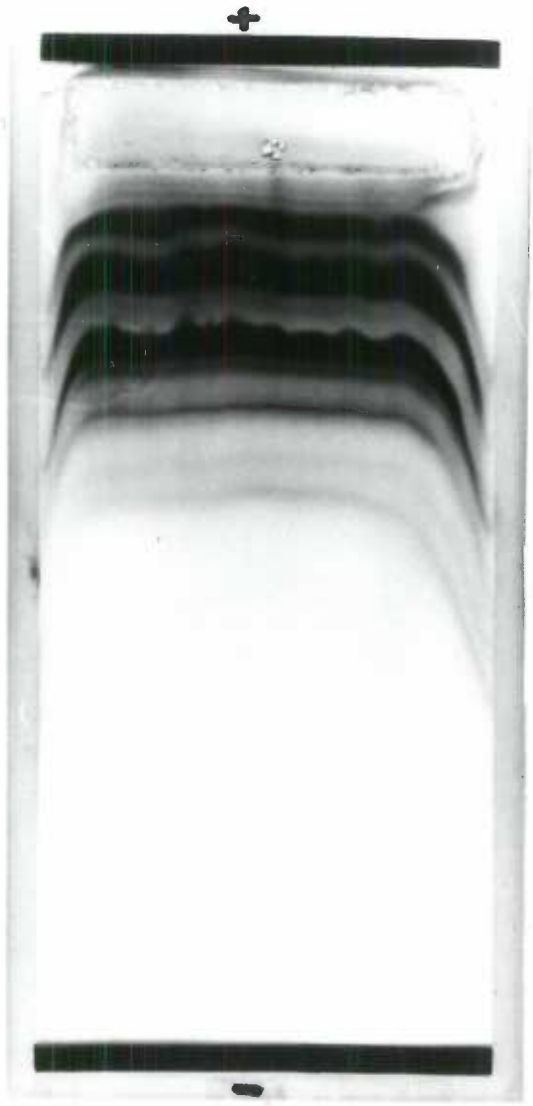

Zeta (ζ) Peptides
ζA
ζB
ζC
XI
ζE
ζF
ζF
X2
X3
ζG
ζI
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVIIa
XVIIb
XVIII
XIX
ABSORBANCE AT 570 nm (—)
pH OF EFFLUENT (- - - -)
2.0
1.0
0.5
0.4
0.2
0.1
0
5
4
3
100
200
300
400
ML EFFLUENT
γT-3
γT-5
γT-3
γT-9
γT-14
γT-15
γT-4
γT-2
γT-12b
γT-12b
γT-1
γT-1
γT-10
γT-10b
γT-6
γT-6
γT-16
γT-7
Gamma (γ) Peptides
γT-9
γT-14
γT-15

Figure 12. Preparative flat bed electrofocusing of hemolyzates from: (a) YLL hydrops and (b) GTT hydrops. Bands #1*-4* of GTT (b) correspond to bands #3-6 of YLL (a) or CFM hydrops (Figure 6).



Band #	Band #
6	
5	(6) 4*
4	(5) 3*
3	(4) 2*
2	(3) 1*
1	
(CFM/YLL)	GTT

(a) (b)

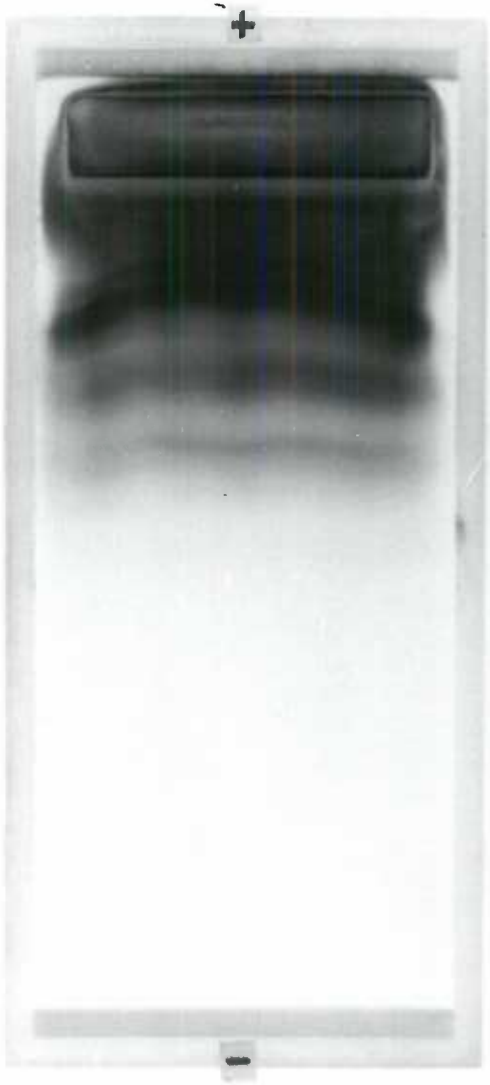


TABLE 6

Percent Area* of the Globin Chains from the HPLC Separation of the Whole Globin from Hydropic Neonates

Serial #	Hydropic Neonate	Amount of Whole Globin Applied (mg)	R.T.* 15 min β?	R.T.* 19 min G_γ	R.T.* 21 min A_γ	R.T.* 23 min ?	R.T.* 37 min ζ
1	CKL	0.5	7	50	36		6
		1.0	6.5	50	37		6
2	GTT	0.5	2	51	24	16	6
		1.0	1	48	25	17	6
3	WM	0.5	6.2	51	37		5.4
		1.0	5.3	49	37		5.8
4	YLL	0.4	11	53	35		2.0
		1.0	10	52	34		3.0
5	TAM	0.5	8.7	62	19		10.6
		1.0	9.0	62	18		10.3
6	CH	0.5	5.6	67	18		5.7
		1.0	5.5	67	18		5.7

*These values represent percentage of the total area due to the absorption at 220 nm and not the actual amount of the globin chain. R.T. Retention Time (HPLC).

isoelectrophoretically focused bands 5 and 3.

II. SEPARATION OF GLOBIN CHAINS

High Pressure Liquid Chromatography -- HPLC conditions used for separating various globin chains are given in Table 3. Figure 13 shows chromatograms of globin chain separations of various hemoglobin lysates by analytical method using a long duration (GCSAL) program of 185 minutes duration. The globin chains are eluted on the basis of their hydrophobicity in the following order: α, β, $^{T}\gamma$, $^{G}\gamma$, $^{A}\gamma$ and ζ. The zeta chain, being very hydrophobic, elutes at 148 minutes. A rapid method was also developed to separate various gamma chains of Hb Bart's (Figure 5A-D) or whole globin preparation from hydropic neonate (Figure 5E), $^{G}\gamma$, $^{A}\gamma$ and ζ chains of Hb Portland I (Figure 5G), and R.T. 15 minutes ($\beta^{?}$) and ζ chains of Hb Portland II (Figure 5F). The assignments of the globin chain was given by separating globin chains of various hemoglobins with known compositions and were further confirmed by examining the tryptic digest of the zones. Figure 4 shows separations of $^{G}\gamma$ and $^{A}\gamma$ chains of Hb Bart's from component I of CKL. These chains elute at 19 and 21 minutes, respectively. The $^{G}\gamma$:$^{A}\gamma$ ratio of 6:40 obtained from the area under these peaks was confirmed by examining the γCB-3 peptide of CKL Bart's globin. Figure 14A shows the separation of 50 mg of cyanogen bromide treated globin of a newborn healthy infant and Figure 14B shows the separation of about 30 mg of cyanogen bromide treated globin of component I of CKL Bart's globin. The HPLC separation procedure for globin chain separation can be analytical (GCSAL or Sh) when using 50-400 µg or preparative (GCSPL or Sh) when loading 0.5-5 mg or more globin on the reverse phase column. Figure 6 shows separation of about 5 mg of component III from CTY

Figure 13. HPLC chromatograms of globin chain separations of hemoglobin lysates by analytical method using a long duration program of 185 min (GCSAL program). Waters μ Bondapak C18 (3.9 mm x 30 cm) reverse phase column was used without any guard column. The separation was made at room temperature at a flow rate of 1.5 ml/min. The composition of solvents 'A' and 'B' is given in the section on HPLC methods. The gradient program is illustrated in Fig. D.

A. 200 μg Hb A ($\alpha_2\beta_2$) isolated from a normal individual.
B. 250 μg Hb Bart's (γ_4) obtained from B #3 by preparative flatbed isoelectricfocusing from CFM hydrops.
C. 300 μg Hb Portland I ($\zeta_2\gamma_2$) obtained from the corresponding Band #5 by preparative flatbed isoelectricfocusing and further purified by starch gel electrophoresis from GTT hydrops.
D. 800 μg cord blood lysate.

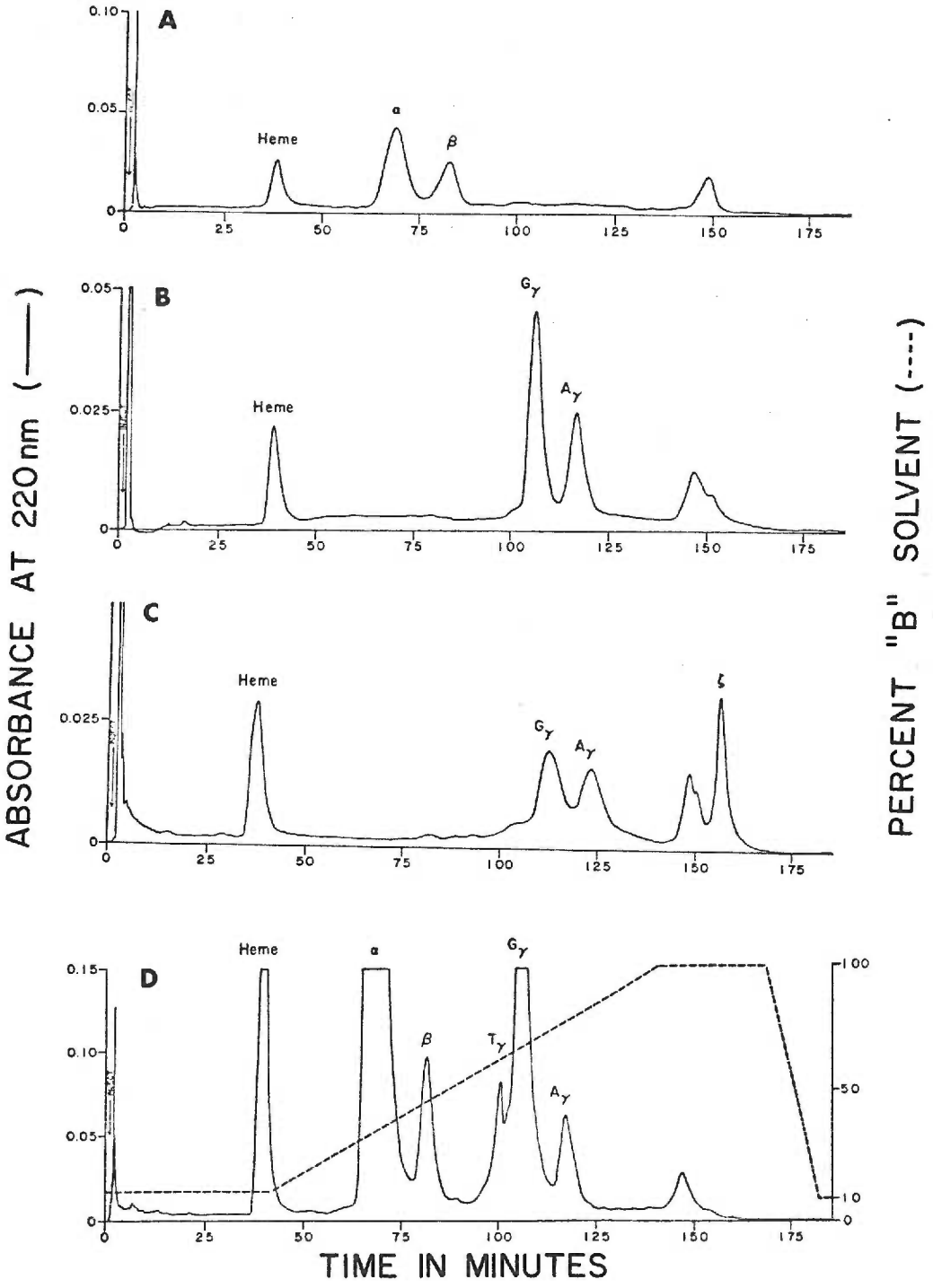
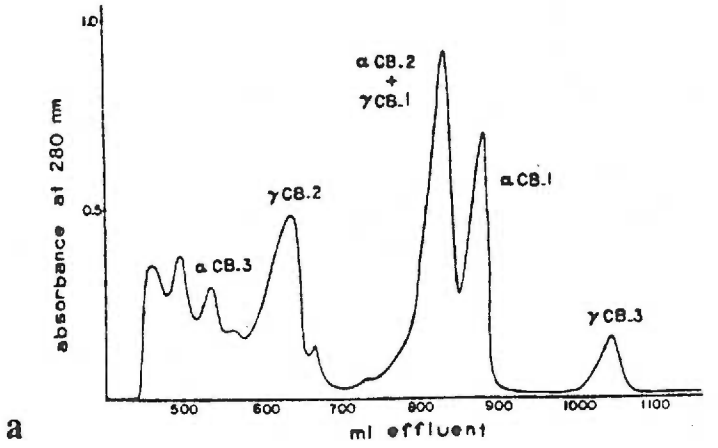
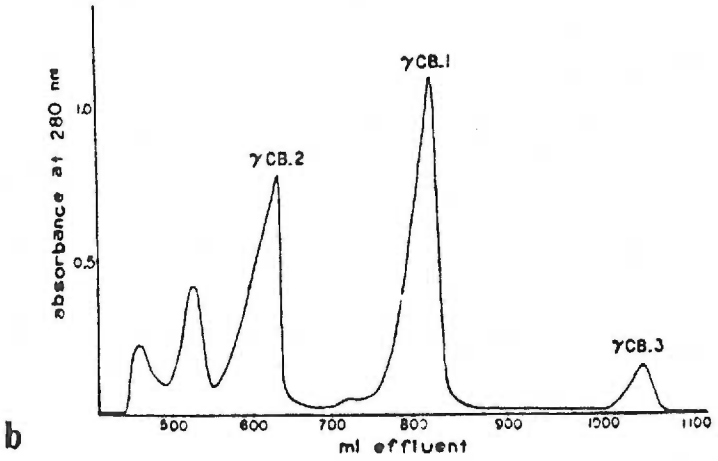

A
Heme
α
β
B
Heme
G_γ
A_γ
C
Heme
G_γ
A_γ
ζ
D
Heme
α
β
T_γ
G_γ
A_γ
ABSORBANCE AT 220nm (——)
PERCENT "B" SOLVENT (----)
TIME IN MINUTES

Figure 14. Chromatogram of cyanogen bromide peptides of globin prepared from hemolysate. Four 1.6 x 160 cm columns of Sephadex G-50 (fine) in series were developed with 1% formic acid. (a) From a healthy newborn; (b) from Hb Bart's (starch gel component I of CKL hydrops).


absorbance at 280 nm
1.0
0.5
αCB.2
+
γCB.1
γCB.2
αCB.1
αCB.3
γCB.3
500
600
700
800
900
1000
1100
ml effluent

a


absorbance at 280 nm
1.0
0.5
γCB.1
γCB.2
γCB.3
500
600
700
800
900
1000
1100
ml effluent

b

hydrops. This separation pattern confirms the assignment of $\zeta_2\beta^?_2$ for Hb Portland II. A similar pattern has also been obtained from component III of CH hydrops. Thus it is concluded that component III from starch gel electrophoresis of hemolyzates from various hydropic neonates is Hb Portland II. Its presence was confirmed in several other neonates with hydrops fetalis due to homozygous alpha-thalassemia (Table 6).

The separation patterns of Hb Portland I and Hb Portland II clearly show that both of these hemoglobins contain zeta chains in addition to their non-zeta chains. The retention time of R.T. 15 of $\beta^?$ globin chain of Hb Portland II appears to be identical to the beta chain of Hb A on the short program. Table 10 outlines some of the evidence that $\beta^?$ chain is in fact a globin-like chain. Since structural studies of $\beta^?$ chain are incomplete, Hb Portland II has been assigned a composition of $\zeta_2\beta^?_2$.

The separated hemoglobin or their purified constituent globin chains were also examined by analytical isoelectricfocusing using 8M urea polyacrylamide gel in the presence of NP 40 (Figure 15) or by electrophoresis on Triton polyacrylamide gels (Figure 16). Figure 17 shows SDS polyacrylamide gel electrophoresis of the purified zeta globin chain from CKL hydrops and Hb A globin.

Countercurrent Distribution Method -- Figure 18A shows separation of 40 mg of Hb F ($\alpha_2\gamma_2$) aminoethylated globin. The alpha chain separated predominantly in the aqueous phase while the gamma chain was in the butanol phase. Figure 18B shows the separation of about 40 mg of Hb Portland I aminoethylated globin from CFM hydrops. This hemoglobin was separated earlier as component II from a fresh hemolyzate by starch gel electrophoresis and had a ζ:γ ratio of 1:1 as determined

Figure 15. Analytical isoelectricfocusing of hydrops globin chains. The globin was made by acid acetone method of the following CKL hydrops samples. (a) Whole hemolyzate, (b) Hb Portland II separated by starch gel electrophoresis, and (c) Hb Bart's separated by starch gel electrophoresis. About 50-100 μg of the globin was dissolved in sample solution and focused in 6-8 pH gel containing 8M urea and NP40. The gel was stained with Coomassie Brilliant Blue, destained and dried.

⊖
⊕
-ζ
G_γ
A_γ
-ORIGIN
WHOLE GLOBIN
PORTLAND
BART'S

Figure 16. Electrophoresis of globin chains on Triton polyacrylamide gel. About 5-20 μg of globin was applied to each well. The gel was stained with Coomassie Brilliant Blue R. (1) Whole globin CKL hydrops, (2) Bart's globin CKL (hemoglobin was obtained from starch gel as component I), (3) purified ζ globin chain from CKL (separated by HPLC procedure at RT 38 min), (4) $^{G}\gamma$ (RT 19), (5) $^{A}\gamma$ (RT 21), (6) Hb F globin, (7) purified α chain, and (8) Hb Bart's globin CFM hydrops (hemoglobin was obtained from B #3 preparative isoelectricfocusing).

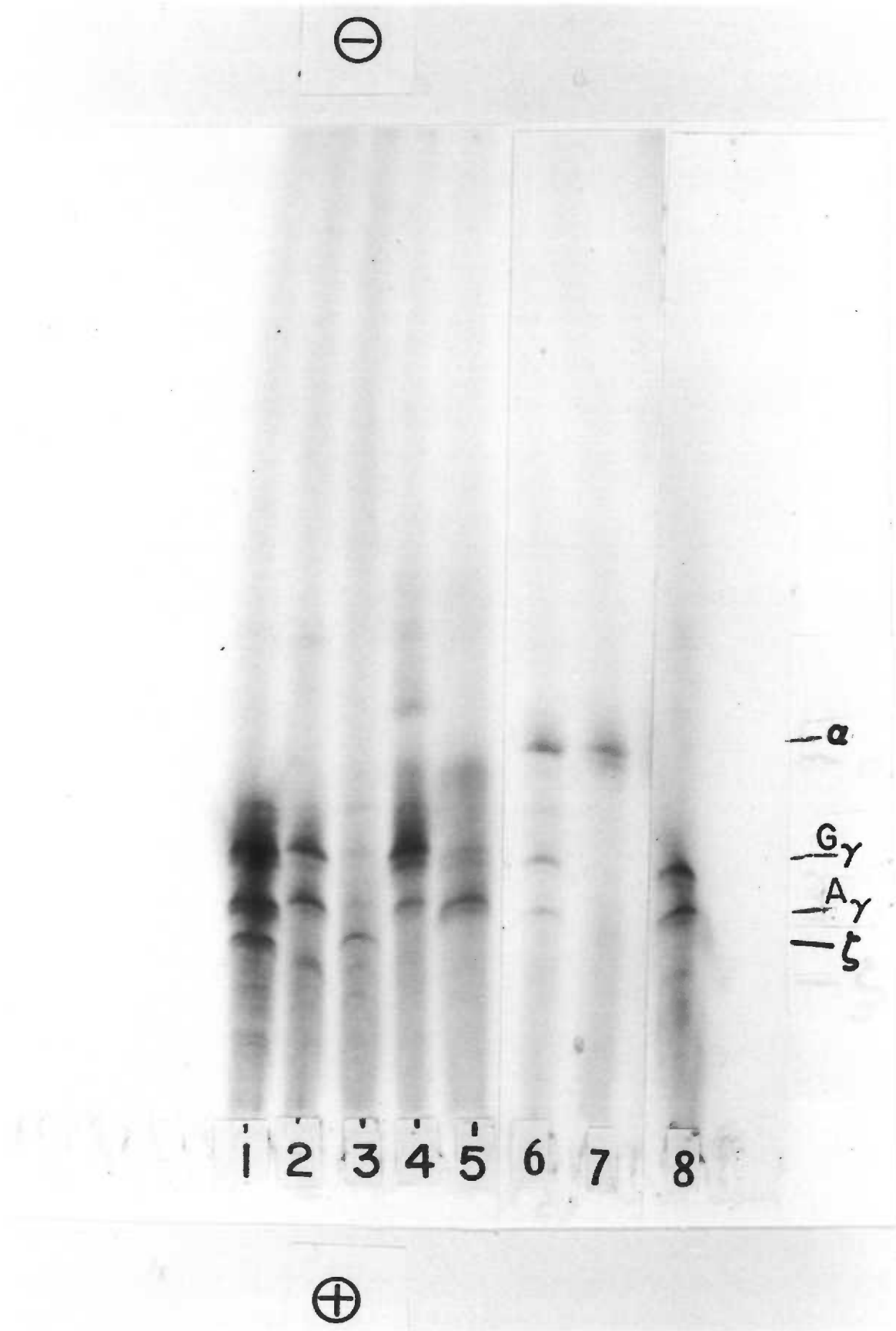
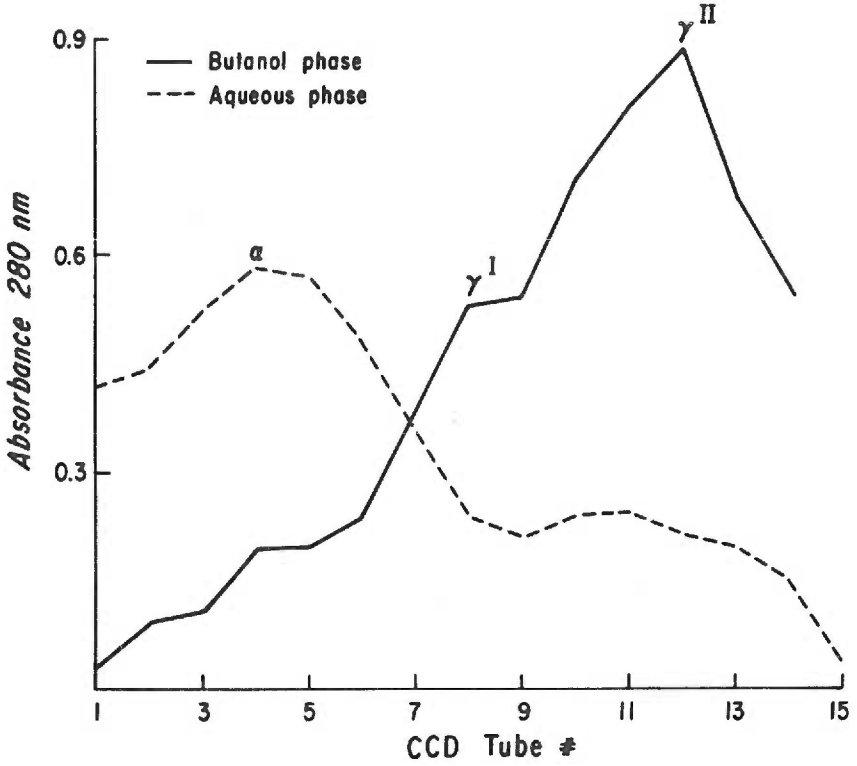

⊖
α
Gγ
Aγ
ζ
1 2 3 4 5 6 7 8
⊕

Figure 17. SDS polyacrylamide gel electrophoresis of purified ζ globin chain and Hb A globin.

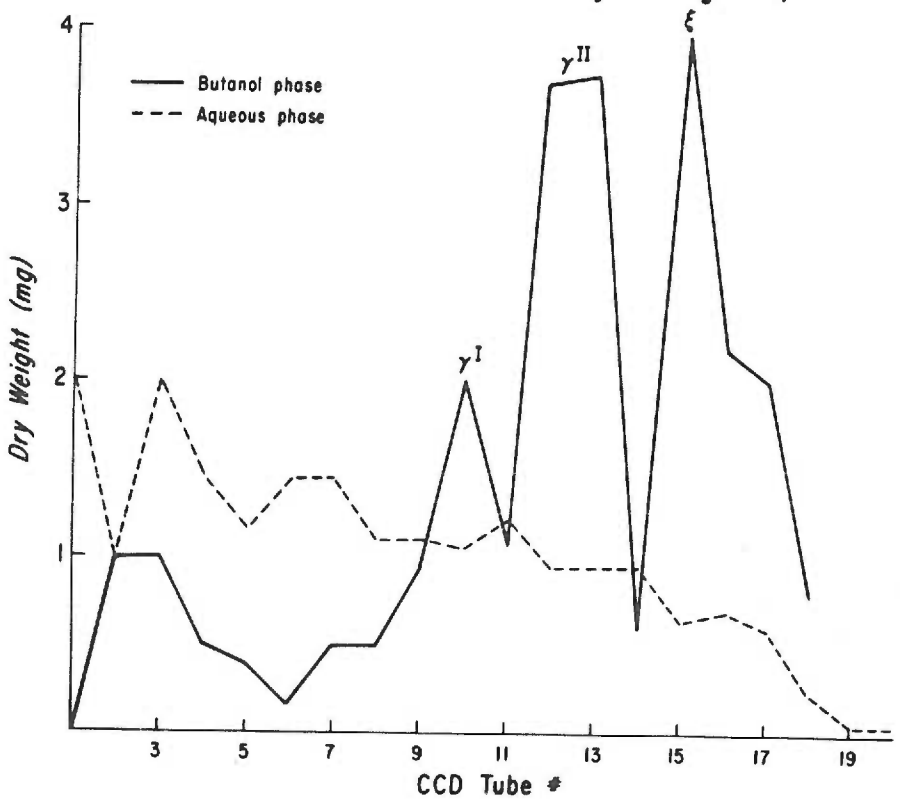
Purified Hb A (Globin)–
Purified ζ (CKL Hydrops)–
⊕
⊖
β
α
ζ

Figure 18. Separation of globin chain by countercurrent distribution method (A) 40 mg Hb F; (B) 40 mg Hb Portland I.

Countercurrent Distribution Pattern of 45 mg Aminoethylated globin chain of Hb F



Countercurrent Distribution Pattern of 42 mg of Hb Portland I (Aminoethylated globin)



by calculating the area under the peaks of peptides XVIII (ζI) and XIX (γT7) obtained from A-5 chromatography of 1 mg of tryptic digest. By using the same calculation procedure, B #5 and B #4 from preparative isoelectric focusing of CFM hydrops were found to contain ζ:γ in the ratios of 1:8 and 1:4, respectively. The whole hemolyzate had 1:20 ratio. From this data it was concluded that preparative isoelectric focusing of relatively old hemolyzates does not provide pure Hb Portland. Further purification of Hb Portland was achieved by starch gel electrophoresis of B #5 and B #4 as shown in Section I of the Results. This approach of separating Hb Portland from Hb Bart's and other components by preparative isoelectric focusing and starch gel electrophoresis provides a protocol for the purification of Hb Portland.

About 80 mg of B #4 and about 80 mg of B #5 of CFM hydrops were separated by the countercurrent distribution method. Based on the separation profile of the zeta chain separation, B #4 contains predominantly zeta-unique and very little zeta-Capp. One difference between zeta-unique and zeta-Capp tryptic peptides is that zeta-unique has no XIX peptide, whereas zeta-Capp has about the same proportion of peptides XVIII and XIX. It is postulated that zeta-unique may be a pure form of zeta, whereas the pattern of zeta-Capp may contain a gamma chain as contaminant.

The zeta chain separated by the countercurrent method from B #5 revealed predominantly the zeta-Capp-like tryptic pattern. Figure 11 shows separation of 40 mg of B #5 tryptic peptides by preparative A-5 chromatography. Examination of tryptic peptides confirms that B #5 contains Hb Portland I in addition to Hb Bart's since the proportion of zeta to gamma is about 1:8. Estimation of the constituent globin chains

for B #4 obtained from preparative isoelectric focusing was not made. However it was found to contain zeta-unique.

III. ISOLATION OF TRYPTIC PEPTIDES

High Pressure Liquid Chromatography -- After globin chain separation the material from each peak was desalted by dialysis and then lyophilyzed. The products of tryptic digestion of aminoethylated or nonaminoethylated forms of purified $\beta^?$ (retention time 15 min), $^{G}\gamma$ (RT: 19), $^{A}\gamma$ (RT: 21), and ζ (RT: 37) globin chains were separated by reverse phase HPLC methods using the short program as outlined in Table 3. The resulting peptides were usually purified further by reverse phase chromatography using different solvent conditions. Experimental details accompany the legends of each corresponding figure and table.

Nomenclature -- Each of the tryptic peptides of the gamma chain is designated by a capital 'T'. The tryptic peptides of gamma chains are numbered in order of their sequence from γT-1 to γT-16. Heterogeneity of gamma tryptic peptides has been shown as $^{G}\gamma$T-15, $^{A}\gamma$T-15, $^{T}\gamma$T-9 or $^{I}\gamma$T-9. The separation of 1 mg of the tryptic peptides of purified, aminoethylated $^{G}\gamma$ chain is shown in Figure 20.

The zeta chain tryptic peptides were designated 'A' through 'J' according to the amino acid composition found by Capp et al. (7) for the zeta chain of Hb Portland I. Additional peptides which have been obtained from the tryptic digest of the purified zeta chain by the present study have been designated K- onward in alphabetical order (Figure 20 and Tables 8 and 9). This nomenclature is adopted because no direct data to date is available for the sequences of the zeta chain or its genome. The separation of the tryptic peptides of $\beta^?$ chain from Hb Portland II of CKL hydrops is shown in Figure 21. A similar pattern was

Figure 19. HPLC separation of the tryptic peptides of aminoethylated G_γ chain on a 4.6 mm x 25 cm Altex Lichrosorb C18 RP column. Sample size was 1 mg in 250 µl of 'A' solvent (10 mM NH_4AC, pH 6.0). The reverse phase column was connected to a guard column containing Vydac RP C18 material and the separation of peptides was made at a flow rate of 1 ml/min.

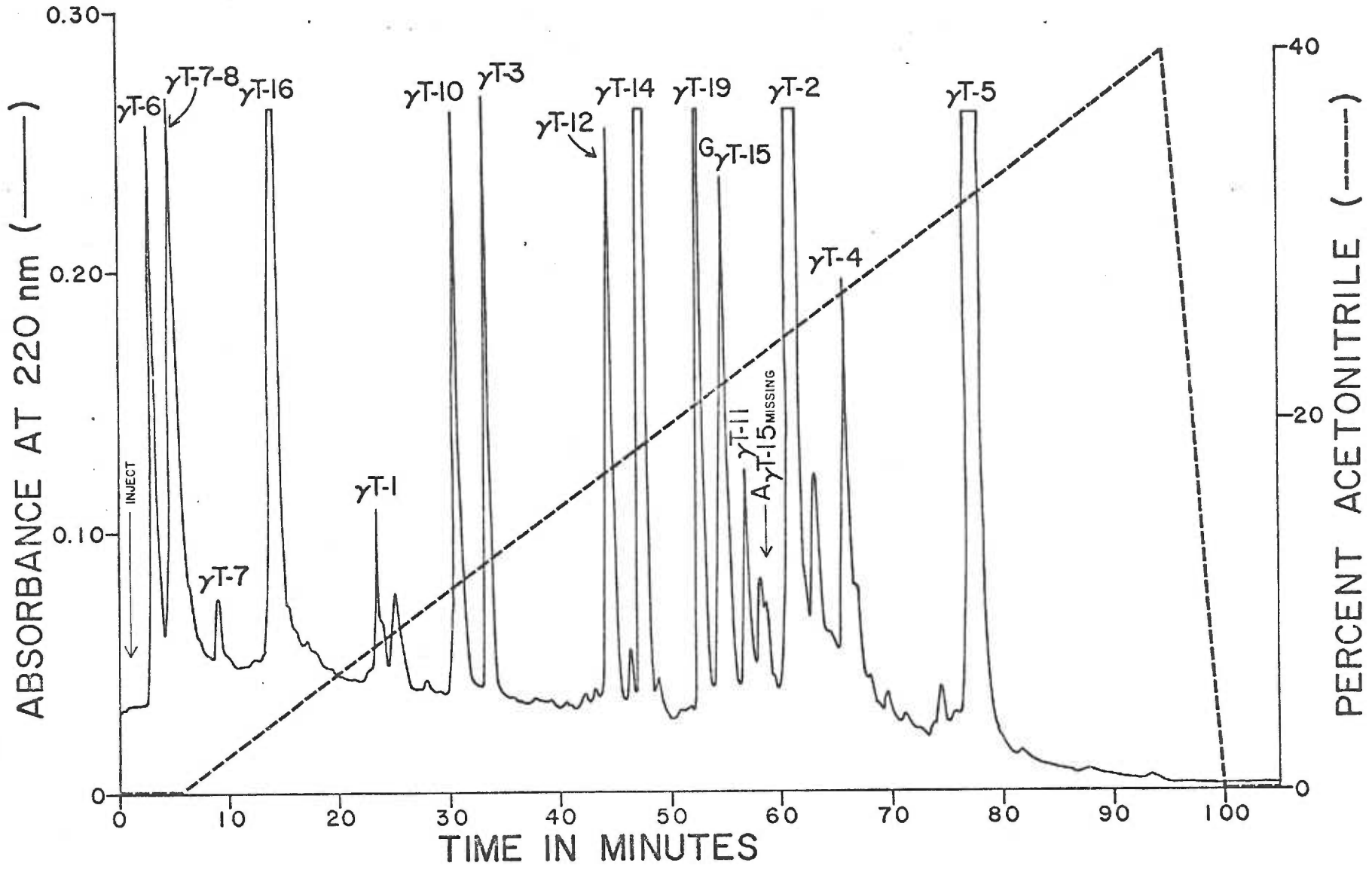

0.30
0.20
0.10
0
ABSORBANCE AT 220 nm (——)
40
20
0
PERCENT ACETONITRILE (- - - -)
γT-6
γT-7-8
γT-16
γT-10
γT-3
γT-12
γT-14
γT-19
γT-2
γT-5
G γT-15
γT-4
γT-11
A γT-15 MISSING
γT-1
γT-7
INJECT
0
10
20
30
40
50
60
70
80
90
100
TIME IN MINUTES

TABLE 7

Amino Acid Analyses of Non-Aminoethylated Tryptic Peptides of ζ Chain from B#5 Preparative Isoelectric Focusing of CFM Hydrops

Zone	I(a)	I(a)	II	VI	VIII	IX	X	XIII(a)	XIII(b)	XVI	XVIII
Lysine	1.0		0.9	1.0		1.4	1.3		1.0		1.0
Histidine					0.9	1.1	1.1		1.0		1.0
Arginine		1.0						1.0		0.9	
Aspartic Acid		1.2		2.1							
Threonine	1.0	3.7	1.0			1.2	1.1	0.9			
Serine	1.0	1.2	2.8		1.0	1.1	1.2				1.0
Glutamic Acid		3.0	1.1		1.2	1.3	1.4	1.0			
Proline						1.0	1.0				
Glycine		1.2									1.0
Alanine		1.4	1.2		1.0						0.9
Cystine											
Valine			1.8								
Methionine											
Isoleucine		1.9			1.9					0.9	
Leucine	1.0	0.6	2.1			2.0	1.9		2.1	1.2	
Tyrosine					1.2						
Phenylalanine			0.8			1.0	1.0				
Tryptophane											
Total Yield/ Residue (nm)	105	68	35	115	63	48	33	129	146	68	198
Designation	ζA	ζB	ζC	X1	ζE	ζF	ζF	X2	X3	ζG	ζI

Figure 20. Separation of the tryptic peptides of about 0.5 mg of aminoethylated ζ chain obtained from Hb Portland II globin of CKL hydrops by HPLC method at RT 38 (min). The separation was made on Altex Lichrosorb C18 RP column according to the conditions given in the legend of Figure 19.

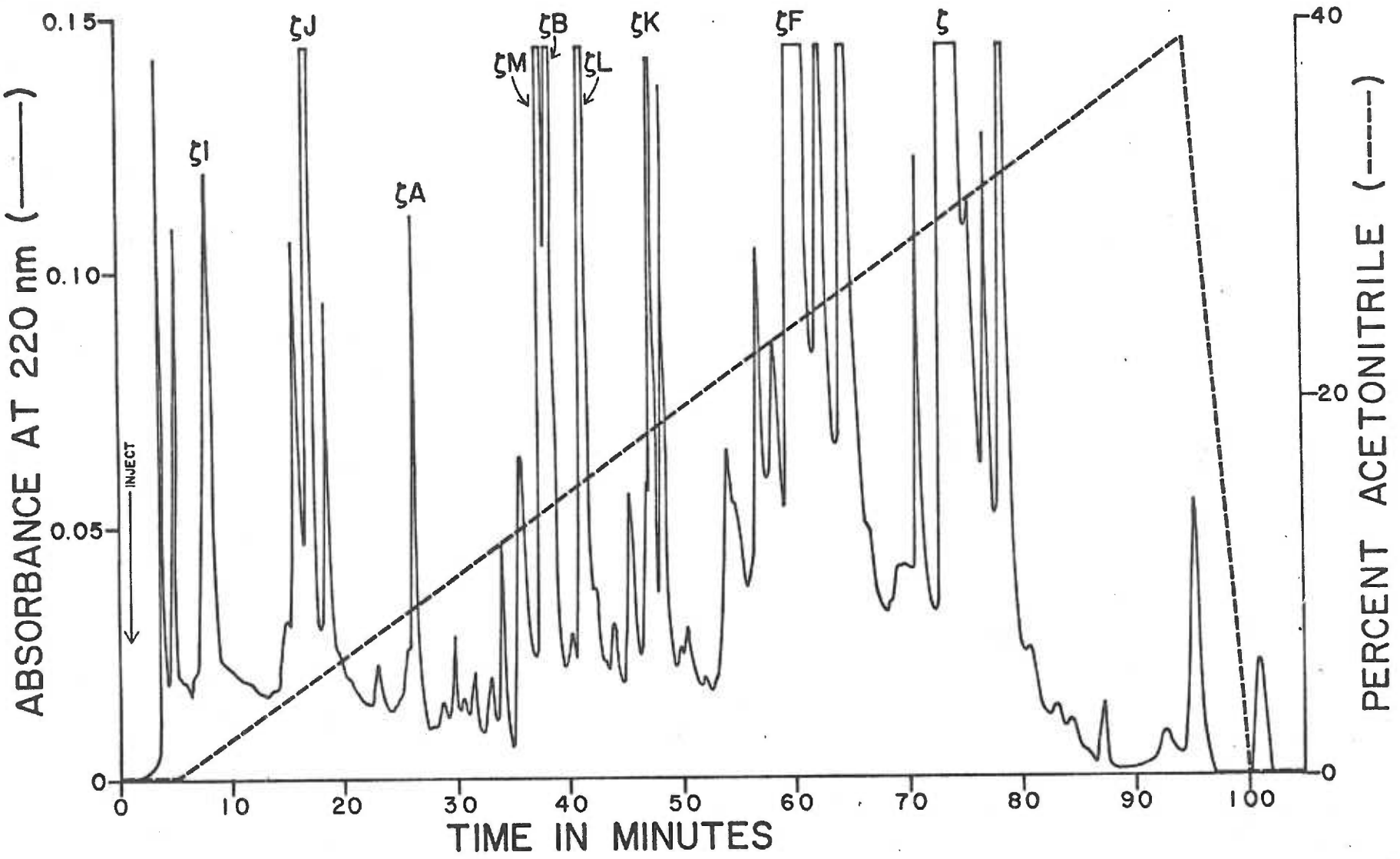

ABSORBANCE AT 220 nm (——)
PERCENT ACETONITRILE (- - - -)
TIME IN MINUTES
INJECT
ζI
ζJ
ζA
ζM
ζB
ζL
ζK
ζF
ζ
0.15
0.10
0.05
0
40
20
0
0
10
20
30
40
50
60
70
80
90
100

Table 8. Rechromatography of the ζ CKL aminoethylated tryptic peptides. (A) Each zone was obtained from tryptic peptide separation of 0.5 mg globin by using NH_4Ac-40% ACN developers, and (B) rechromatography by KH_2PO_4-60% ACN developers. The designations of purified peptides are based on amino acid composition. Some of the peptides designated from A-J are amongst those reported earlier by Capp et al. (7,8), whereas peptides with designations from K- onward represent additional ζ peptides.

TABLE 8

CHROMATOGRAPHY "A"	CHROMATOGRAPHY "B"
Retention Time (R.T.) of the Zone in Minutes	R.T. of the Zones and the Designation of the ζ Peptide (A-M)
3.9	3.5 (Mixture)
4.7	4.2 (Mixture)
7.6	5.1 (ζI)
17.0	12.0 (ζJ)
26.3	26.9 (ζA)
37.8	32.1 (ζM)
38.6	32.1 (ζM), 38.7 (ζB)
41.4	36.01 (ζL)
47.5	40.38 (ζK)
48.5	41.9 (none)
58.4	41.6 (none)
60.3	36.2 (ζF)
73.8	48.3 (Mixture)

Mixture = More than one peptide; could not be separated by rechromatography

TABLE 9

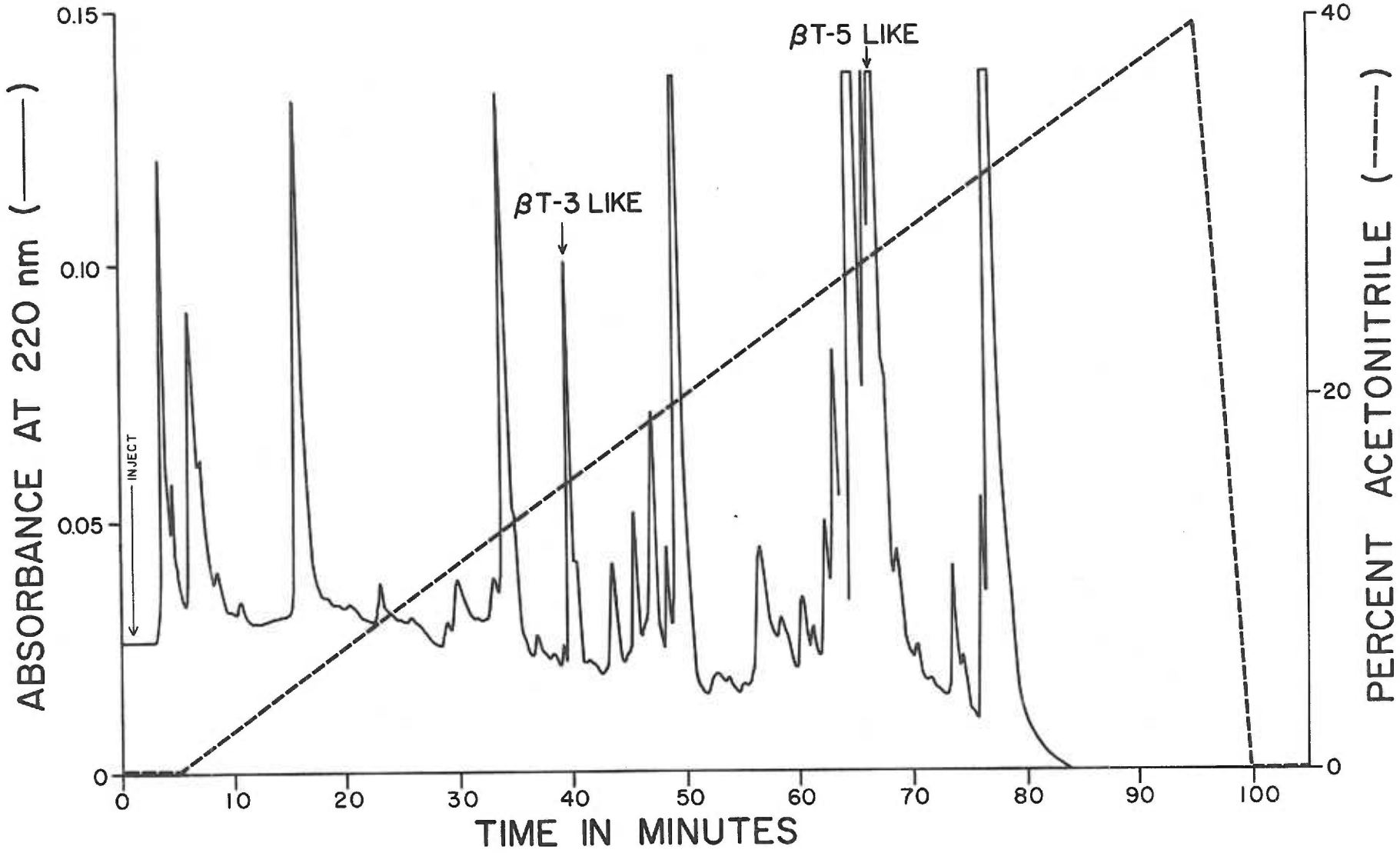
Amino Acid Analyses of Tryptic Peptides from Aminoethylated ζ Chain of CKL Hydrops Separated by HPLC Method (R.T. 37 min)

R.T.*	7.6	17.0	26.3	37.8	38.8	41.4	47.5	60.3	73.8
Lysine	1.0		1.01	1.16		1.0	1.0	0.81	1.1
Histidine	0.93							1.09	2.2
Arginine		1.0			1.0				1.0
Aspartic Acid				1.0	1.01	1.84	2.08		1.01
Threonine			1.0		3.52			1.0	2.3
Serine	1.01		1.0		1.16		1.84	1.18	3.9
Glutamic Acid					2.92			1.20	2.1
Proline						0.84		1.01	1.9
Glycine	1.09			1.13	1.21		2.10		1.3
Alanine	0.95			2.98	1.10		1.08		1.1
Cystine									
Valine**				2.43		1.8			2.5
Methionine									
Isoleucine					2.0		2.22		
Leucine			0.94		1.05		1.17	2.2	4.1
Tyrosine		0.97							0.95
Phenylalanine						0.84		0.98	2.84
Tryptophane									
Total Yield/ Residue (nm)	10.4	16.3	16.4	14.5	12.1	15.6	11.6	13.2	8.4
Designation	ζI	ζJ	ζA	ζM	ζB	ζL	ζK	ζF	ζMix

*Retention times (minutes) were obtained by using ammonium-acetate-acetonitrile gradient program. The amino acid analysis of each zone was obtained after rechromatography by using phosphate-acetonitrile gradient program.

**22 hr hydrolysis only.

Figure 21. Separation of the tryptic peptides of about 0.5 µg of aminoethylated X-chain obtained from Hb Portland II globin of CKL hydrops by HPLC method at RT 15 (min). The separation was made on Altex Lichrosorb C18 RP column according to the conditions given in the legend of Figure 19.


0.15
0.10
0.05
0
ABSORBANCE AT 220 nm (——)
INJECT
βT-3 LIKE
βT-5 LIKE
40
20
0
PERCENT ACETONITRILE (- - - -)
0
10
20
30
40
50
60
70
80
90
100
TIME IN MINUTES

also obtained from the tryptic digest of $\beta^?$ chain from Hb Portland II of CTY hydrops. Table 10 shows retention times and amino acid compositions of some of the tryptic peptides analyzed from $\beta^?$ chain of CKL and CTY hydrops. The same table also shows the amino acid composition of some of the $\beta^?$ tryptic peptides of Hb Portland II from CKL and CTY hydrops. Separation of tryptic peptide of about 1 mg aminoethylated beta-globin chain obtained from purified Hb A is shown in Figure 22.

Aminex A-5 Ion Exchange Peptide Chromatography -- Forty milligrams of band #5 separated by preparative isoelectric focusing from CFM hydrops and containing a $\zeta:\gamma$ ratio of 1:8 was digested with 0.4 mg of TPCK treated trypsin at 37°C, pH 8.5 for 2 hrs. The non-aminoethylated tryptic peptides were separated as described in the legend to Figure 11. Rechromatography of one half of the amount of most of the zones was done on Dowex 50 WX4 resin. The other half was rechromatographed by the HPLC method. The zones obtained by ion exchange chromatography are numbered I to XIX as shown in Figure 11. The amino acid composition of the purified tryptic peptides of the zeta from CFM hydrops are shown in Table 7. All peptides except ζD and ζH were found to elute on A-5 chromatography as well as on rechromatography at positions reported by Capp et al. (7). Their compositions were also as reported earlier. Rechromatography by HPLC of tryptic peptides from CFM hydrops provided additional information about the zeta and gamma chains which was used in identifying various tryptic peptides from other hydrops (Table 11). A cysteinyl peptide (ζH) could not be found. This may have been because the sample used for tryptic peptide separation was nonaminoethylated, whereas the Capp et al. (7) preparation was aminoethylated. No tryptic peptide with a composition of ζD reported by

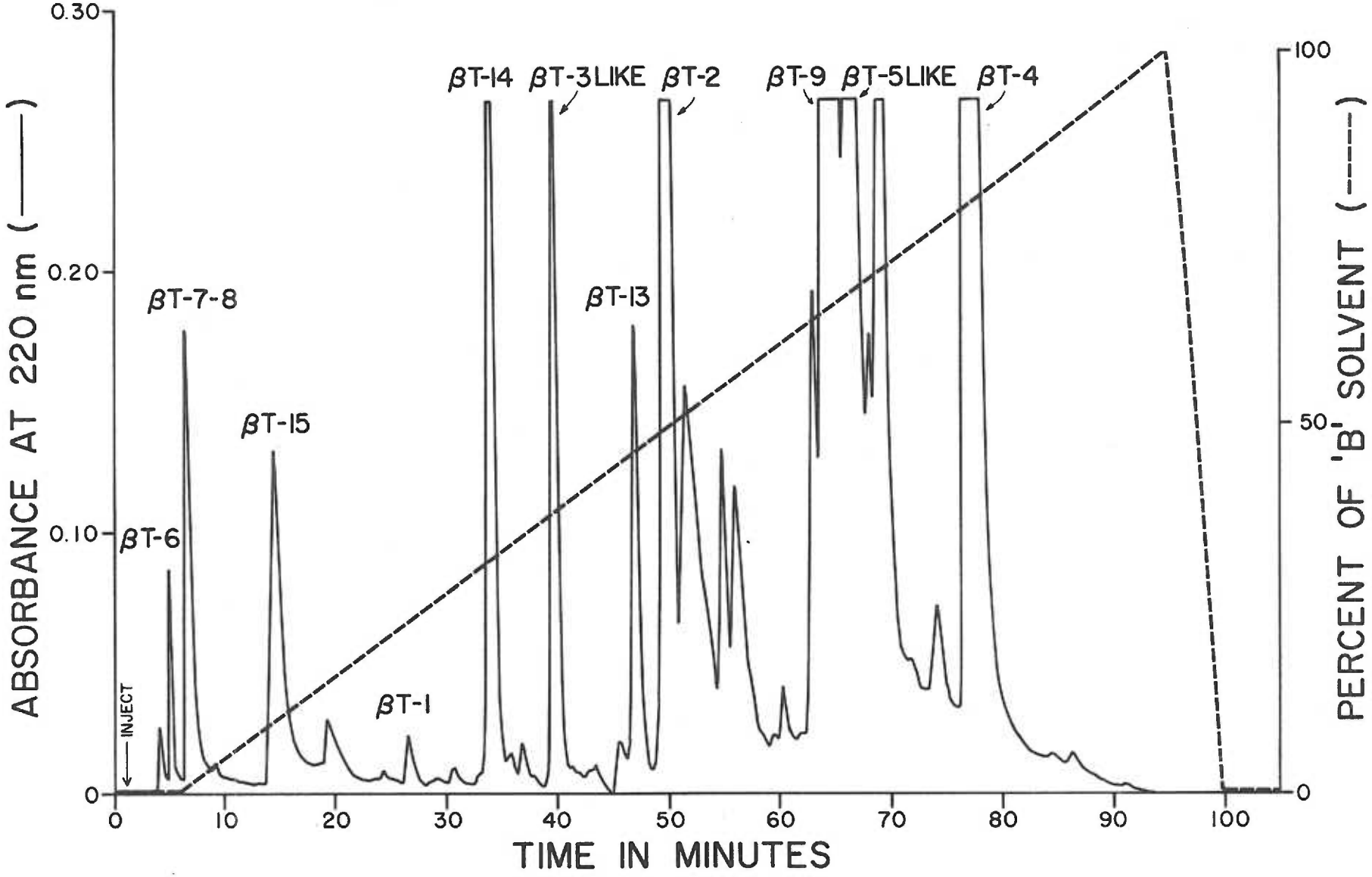
TABLE 10

Amino Acid Composition of Two Tryptic Peptides from $\beta^?$ Globin Chains of Hb Portland II ($\zeta_2\beta_2^?$) from CTY and CKL Hydropic Infants

	(A) T-3-LIKE PEPTIDE						(B) T-5-LIKE PEPTIDE				
Amino Acid	R.T. 39.7 CKL	R.T. 39.9 CTY	R.T. 39.7 βT-3	R.T. 33.2 γT-3	εT-3	δT-3	R.T. 66.8 CTY*	R.T. 67.4 βT-5	R.T. 77.0 γT-5	εT-5	δT-5
Lysine							1.0(1)	1	1	1	1
Arginine	1.0(1)	1.0(1)	1	1	1	1					
Aspartic Acid	2.1(2)	1.9(2)	2	2	1	2	2.7(3)	3	3	3	3
Threonine	0	0	0	1	0	0	0.9(1)	1	0	0	0
Serine							1.9(2)	2	4	3	4
Glutamic Acid	2.0(2)	2.2(2)	2	2	3	1	0.9(1)	1	0	1	0
Proline							1.8(2)	2	1	2	2
Glycine	3.1(3)	2.9(3)	3	3	3	3	2.3(2)	2	2	2	2
Alanine	1.0(1)	1.2(1)	1	1	2	2	1.1(1)	1	2	1	1
Valine**	2.6(3)	2.6(3)	3	2	1	3	1.2(1)	1	0	1	0
Methionine	0	0	0	0	1	0	0.7(1)	1	1	1	0
Isoleucine							0	0	1	0	1
Leucine	1.0(1)	1.0(1)	1	1	1	1	1.1(1)	1	1	1	2
Phenylalanine							2.9(3)	3	3	3	3
Av. Residue (nm)/Total	6.3(13)	3.8(13)	13	13	13	13	4.1(19)	19	19	19	19

*Peptide zone (R.T. 67.1) from CKL hydrops $\beta^?$ chain had identical amino acid composition.
**22 hr acid hydrolyzate

Figure 22. Separation of the tryptic peptides of about 1 mg of aminoethylated β-globin chain obtained from purified Hb A sample of a normal individual. The separation was made on Altex Lichrosorb C18 RP column according to the conditions given in the legend of Figure 19.


0.30
0.20
0.10
0
ABSORBANCE AT 220 nm (——)
βT-14
βT-3LIKE
βT-2
βT-9
βT-5LIKE
βT-4
βT-7-8
βT-13
βT-15
βT-6
βT-1
INJECT
100
50
0
PERCENT OF 'B' SOLVENT (----)
0
10
20
30
40
50
60
70
80
90
100
TIME IN MINUTES

Capp et al. (7) could be found. Additional peptides not reported earlier are designated X1, X2 and X3. The composition of these peptides is shown in Table 7.

DISCUSSION

The results obtained by electrophoresis and analysis of the constituent globin chains of all the components from hemolyzates of blood from infants with hydrops fetalis indicate a total absence of alpha-chain production. The results presented here also provide clear evidence that in addition to Hb Portland I ($\zeta_2\gamma_2$) identified earlier by Capp et al. (7) most of the neonates with homozygous alpha-thalassemia produce another zeta chain containing hemoglobin with the globin composition of $\zeta_2\beta^?_2$. The preliminary studies on globin chain with R.T. 15 (mins) have been identified here as $\beta^?$ due to the presence of βT-3 tryptic peptides and similarity of tryptic peptide patterns of $\beta^?$ and β chain of Hb A. These findings have ruled out the possibility of $\beta^?$ being the epsilon chain (i.e., Hb Gower I, $\zeta_2\varepsilon_2$) and hence this new zeta containing hemoglobin has been tentatively designated by the name of Hb Portland II ($\zeta_2\beta^?_2$).

All the ten infants with homozygous alpha-thalassemia that have been studied so far have significant to detectable amounts of zeta chain and most also have $\beta^?$ globin chain in their cord blood. This conclusion that all these neonates were producing, in addition to predominant amounts of Hb Bart's, either Hb Portland I ($\zeta_2\gamma_2$) or Hb Portland II ($\zeta_2\beta^?_2$) or both of these hemoglobins in detectable amounts is based upon determinations by starch gel electrophoresis and HPLC methods. The presence of Hb Portland II in some hydropic neonates has been confirmed by separating this hemoglobin as component III on starch gel followed by subsequent analysis of the globin chains from CTY (Figure 6), YLL and CH hydrops. CKL hydrops which did not have any significant amount of Hb Portland I contained about 15% of Hb Portland

II. The cord blood of another hydropic neonate GTT did not have any detectable amount of $\beta^?$ chain in the whole globin preparation or in any of the blood components but rather contained only Hb Portland I in addition to the predominant amount of Hb Bart's. Similar results from CFM hydrops were also obtained.

Hb Portland I ($\zeta_2\gamma_2$) was separated as component II on starch gel. It moves faster than Hb A but slower than Hb Bart's (Figure 3). A hemoglobin with identical mobility and composition was first characterized by Capp et al. (7) as Hb Portland I from a female Chinese infant having multiple congenital anamolies by starch gel electrophoresis with Tris-EDTA-borate at pH 8.15. Later on it was found that the embryonic hemoglobin reported by Kaltsoya et al. (61) as Hb X in three human embryos during early development had an electrophoretic behavior similar to Hb Portland I. Weatherall et al. (18) also separated Hb X with mobility identical to Hb Portland I by starch gel electrophoresis using Tris-EDTA-borate at pH 8.6 from three hydropic neonates with homozygous alpha-thalassemia. Rutherford et al. (33) identified a hemoglobin obtained from K562 human leukemic cell lines induced with hemin that had a mobility identical to Hb Portland I. This hemoglobin was found to contain zeta and gamma globin chains as revealed by the Triton X-100 electrophoresis method of Alter et al. (45). Later Pressley (62) also separated Hb Portland I from the cord blood of a Greek infant with Hb Bart's hydrops fetalis by using starch gel electrophoresis. The results of all these investigations clearly prove that Hb Portland I is produced in a variety of conditions and can be separated by starch gel electrophoresis as component II.

However, Hb X separated by Todd et al. (19) by starch gel

electrophoresis using Tris-EDTA-borate buffer at pH 8.6 might be a mixture of Hb Portland I and Hb Portland II. The electrophoretic mobility of their Hb X appears to be identical to Hb Portland II (i.e., component III as shown in Figure 3). This can be confirmed further by the compositional analysis of Hb X which Todd et al. found consisted of gamma chains, zeta chains and beta chains. The possibility of R.T. 15 mins ($\beta^?$) chain of Hb Portland II being beta globin chains is examined in a subsequent section on tryptic peptides.

Hemoglobin components with the mobility of Hb Portland I (starch gel component II) and Hb Portland II (starch gel component III) were also separated by Pootrakul in a Thailand survey of hemoglobins (63) from a neonate with Hb Bart's hydrops. In that survey a hemoglobin component separated by starch gel electrophoresis with the mobility identical to Hb Portland II was assumed to be Hb F ($\alpha_2\gamma_2$). The presence of Hb F as any fraction in hydropic blood must be incorrect since hydrops fetalis results from complete deletion of alpha genes (27). It is, therefore, proposed that the hemoglobin component identified by Pootrakul as Hb F was in fact Hb Portland II. In conclusion, both Hb Portland I and Hb Portland II have previously been separated by various investigators from hydropic neonates.

Hb Portland I is now recognized as a normal embryonic human hemoglobin. Its presence in trace amounts in normal newborn babies (31) and significant proportions during early embryonic life has been reported by several investigators. It is postulated that Hb Portland II (assuming its structure to be $\zeta_2\beta_2$) may not exist in normal newborn neonates since these infants produce significant amounts of alpha-chain and the affinity of alpha-chain for adult beta-chain may be greater than

zeta-chain for beta. This may also be true since the zeta-chain in normal newborn neonates is produced in very small amounts and thus would be difficult to detect in trace amounts even if Hb Portland II is produced. It can therefore be argued that Hb Portland II can only be produced in special conditions where the globin chain synthesis of zeta and beta is active and synthesis of the alpha chain is either absent or remarkably reduced.

Since hydropic neonates are deficient in alpha-chain and they continue synthesizing zeta-chain, it is postulated that during the early embryonic life these fetuses might produce Hb Gower I ($\zeta_2\varepsilon_2$) followed by Hb Portland I ($\zeta_2\gamma_2$). From the fact that CKL hydrops produced significant amounts of Hb Portland II ($\zeta_2\beta^?_2$) in addition to large amounts of Hb Bart's (γ_4) it is postulated that perhaps this infant was producing adult beta-chain. The fact that there was no Hb Portland I produced by this hydrops in significant amounts leads to the suggestion that the beta-chain may have greater affinity for the zeta than gamma-chain has for zeta chain. Based on this assumption it is also speculated that those hydropic neonates which are born closer to full term pregnancy or have active adult beta-chain synthesis may be producing Hb Portland II. Hydropic infants which are born premature are therefore believed to produce predominant amounts of Hb Portland I since little or no beta-chain is synthesized actively prior to 30 weeks of intrauterine life of the fetus.

The presence of Hb Portlands I and II in some neonates can be explained by assuming preferential affinity of beta for zeta as compared to gamma for zeta. Such infants appear to produce proportionally more zeta than beta chains. Thus the excess zeta is bound to gamma to

produce Hb Portland I. Hydropic infants which produce Hb H probably do not produce any Hb Portland I. In this case excess beta-chain exists as Hb H (β_4) while all zeta exists as Hb Portland II ($\zeta_2\beta^?_2$). The presence of Hb Portland II in pathological states other than hydrops fetalis remains to be shown.

Starch gel component IV has not been investigated yet. It is unlikely to be Hb Gower I ($\zeta_2\varepsilon_2$) since epsilon-globin synthesis is active during early embryonic life and most of the hydropic neonates included in this study were born close to full term pregnancy. Moreover, based on electrophoretic mobility of component IV on starch gel, it is unlikely to be Gower I since Gower I moves slower than Hb A2, whereas component IV moves faster than Hb A2. It is postulated that component IV may have a composition of $\zeta_2\delta_2$ and may be designated as Hb Portland III. The existence of component IV as a zeta and delta containing hemoglobin component would be more consistent with the adult delta-chain production closer to full term pregnancy after the adult beta-chain synthesis is fully activated. Also components III or IV are unlikely to be Hb X ($\gamma_2\varepsilon_2$). A hemoglobin with this composition has recently been reported in some embryonic neonates and in human K562 cell lines when induced by hemin (33).

The presence of Hb Portland I and Hb Portland II suggests that zeta is alpha-like in its association with non alpha-chains. The alpha-chain forms similar products with gamma and beta chains during fetal development as fetal type Hb F ($\alpha_2\gamma_2$) and adult Hb A ($\alpha_2\beta_2$). Based on these findings the existence of Hb Portland III ($\zeta_2\delta_2$) cannot be ruled out since the proposed tetramer would correspond to Hb A2 ($\alpha_2\delta_2$). The nature of the globin chains of Hb Bart's (component I), Hb Portland I

(component II) and Hb Portland II (component III) was investigated by converting pure hemoglobin components to globin by cold, acidified acetone, separation of the chains by a newly developed high pressure liquid chromatography method, aminoethylation with ethylenimine and digestion with trypsin, followed by A-5 chromatography or separation of tryptic peptides by HPLC methods. The resulting peptides were subjected to amino acid analysis after rechromatography.

Analytical and preparative, as well as short and long duration HPLC programs employing analytical or preparative μ Bondapak C-18 reverse phase columns and using appropriate solvents and gradient programs have been developed (Table 3). These programs and HPLC conditions permit the separation of 50 μg to 5 mg of globin/lysate. Figure 4 shows the procedure for cleaning the reverse phase column. This procedure has been found to be very useful in cleaning the column by actually dislodging the leftover sample material as can be seen in Figure 4A-D. Component I Hb Bart's of CKL hydrops provided $^{G}\gamma:^{A}\gamma$ of 60:40 which was confirmed by separating γ-CB3 peptide from CNBr treated Hb Bart's by gel permeation column (G50F) and determining the ratios by amino acid composition. This procedure provides a separation of several mgs of globin chain from Hb Bart's or Hb Portland by using preparative column in less than 45 mins. These programs allow the separation of various γ chains, ζ and β? (R.T. 15 mins) from the hemoglobin components or globin samples of hydropic neonates as shown in Figures 4-6. In addition, globin chains of normal cord blood samples can be quantitated by using 200-400 μg of whole hemolyzate (Figure 13D). These separations provide good evidence that almost all types of human globin chains can be separated in pure form as revealed by the structural analysis of each

of the zones (Figures 19-21).

The zeta chain of Hb Portland I and Hb Portland II is very hydrophobic as revealed by its elution position on HPLC. For this reason, it can be explained now, why the previous investigators were unable to separate zeta chain by any of the chromatographic procedures except for Capp et al. (7) who purified zeta chain by a countercurrent distribution method. In essence for the first time a chromatographic method to separate zeta-chain from other globin chains has been developed. This procedure, therefore, can be very useful in quantitating the relative proportion of the zeta-chain or any other known human globin chain in various physiological states or at different stages of human development. It is also a useful method for the preparative isolation of the zeta or other globin chains.

Shelton et al. (54) and Congote et al. (64) were the first to develop techniques of HPLC for the separation of certain hemoglobin chains such as the α and β chains of Hb A and α, $^{G}\gamma$ and $^{A}\gamma$ chains of fetal hemoglobin. Shimieu et al. (65) and Huisman and Wilson (55) introduced some modifications in the procedure of Shelton, allowing the resolution of three gamma chains (i.e., the $^{G}\gamma$ with Ile at position 75 and Gly at position 136; the $^{A}\gamma$ chain with 75 Ile and 136 Ala; and the $^{A}\gamma^{T}$ chain with 75 Thr and 136 Ala). Huisman et al. (66) further demonstrated the versatility of HPLC methods by quantitating the gamma chain heterogeneity of Hb F of numerous patients with sickle cell anemia or Hb S-heterogeneity persistance of Hb F (S-HPFH) using a 180 minute duration program.

Figure 13 shows separation of globin chains of Hb A, Hb Bart's, Hb Portland I and cord blood hemolyzate by a gradient

essentially identical to that of Huisman et al. (55). By comparing the globin chain pattern of embryonic hemoglobins obtained by Huisman et al. (55) with zeta-chain separation of Hb Portland I and II it can be concluded that their assignment of zeta-chain eluting slightly ahead of $^{A}\gamma$ chain may be incorrect. This is particularly true since their assignment is apparently based on the assumption that a new zone from a mixture of hemin induced K562 cells must be a zeta chain. Furthermore, their claim of zeta's position is not based on actual structural analysis. Figures 5, 6 and 13 provide clear evidence that the zeta chain is very hydrophobic and does not elute until solvent "B" = 100%; i.e., well after the elution of $^{A}\gamma$ chain. This is further supported by examining the tryptic peptides of the zone designated in the present work as zeta-chain (Figure 20). One way to explain the differences in the elution position of zeta chains from the same type of column and developer condition as described by Huisman et al. and by the present study is to assume the existence of two kinds of zeta polypeptides. Lauer et al. (12) have identified two zeta genes -- ζ1(3') and ζ2(5') -- in human genomic DNA. These workers have also completed partial nucleotide sequence of genomic DNA from the ζ1(3') gene. Since the sequenced portion of the fragment corresponded with partial amino acid sequence of the tryptic peptides of zeta globin polypeptide determined by Clegg (unpublished work as cited by Lauer et al.), Lauer et al. argue that ζ1 ought to be a functional gene. This conclusion can be questioned since partial sequence data of ζ1 cannot be used to support the conclusion that a gene is functional, particularly when the supposed product (ζ globin) of the gene (in this case ζ1) has also been partially sequenced. The ζ globin [partially sequenced by Clegg (12)] can also be

the product of ζ2(5') and not ζ1(3') gene.

Convincing evidence that ζ2(5') may be a functional gene was provided by Pressley (62) who did genetic mapping of the zeta gene in two infants with hydrops fetalis. DNA from a Thai infant lacked the ψα1 gene (a nonfunctional gene) and both the alpha genes; however, both the zeta genes were present. A Greek infant's DNA was found to lack the ζ1(3') gene. Because the Hb Portland I was still synthesized in the Greek infant, Pressley et al. concluded that ζ2(5') gene identified earlier by Lauer et al. must be a functional gene. Based on these observations, instead of concluding that ζ2(5') is functional, Lauer et al. (12) concluded that both the ζ1 and ζ2 are functional genes. Recent structural studies of Proudfoot et al. (67) show a termination codon (UAG) at amino acid position number 6 of the ζ1(3') gene which should make it nonfunctional. Thus at present only one functional zeta gene (ζ2) is known. Based on these observations, zeta chain eluted at R.T. (retention time) 37 minutes on a short duration program (Figure 5) or at R.T. 60 minutes on a long duration HPLC program (Figure 13C) is postulated to be the product of the ζ2(5') locus. Unless there is another active locus for the zeta-chain production, the zeta zone claimed by Huisman et al. to be just ahead of $^{A}\gamma$ chain must be an artifact. Even if ζ2(5') were present in the samples (K562 cell lines) that Huisman et al. had used, their gradient program does not seem to have eluted the zeta chain. This can be seen from the HPLC patterns of zone 3 and 4 of their figure 8 which they had recovered by chromatography from K562 cells (55).

That the zeta chain is present in human K562 cells has been shown by Rutherford et al. (33) by using Alter's Triton X100 gel

electrophoresis method (45). Rutherford et al. were able to separate Hb Gower I ($\zeta_2\varepsilon_2$) and Hb Portland I ($\zeta_2\gamma_2$) from hemin induced K562 cell lines by starch gel electrophoresis and then demonstrate the separation of the constituent globin chains by gel electrophoresis method. Using identical electrophoresis conditions (Figure 16) the purified zeta-chain of CKL hydrops by HPLC method was found to have electrophoretic mobility identical to that reported by Rutherford et al. Based on these observations it is postulated that all the zeta-containing hemoglobin components (i.e., Gower I, Portland I and Portland II) may contain identical zeta polypeptides (5'ζ2). In conclusion, at present only one zeta-chain gene (5'ζ2) and one epsilon-chain gene are believed to be functional.

The separation of tryptic peptides by HPLC methods provides a new dimension to the peptide chromatography. The reverse phase column when used with appropriate developers acts like an adsorption column and separates the peptides on the basis of their polarity. In other words the reverse phase HPLC columns separate the peptides on the basis of their hydrophobicity rather than the charge or size. These separation methods are very useful in examining the heterogeneity, particularly the gamma tryptic peptides such as threonyl or isoleucyl peptides of γT-9 or glycyl or alanyl peptides of γT-15.

Figure 20 provides the evidence for the separation of tryptic peptides $^{G}\gamma$T-15 and $^{I}\gamma$T-9 from $^{G}\gamma$ globin chain. The $^{G}\gamma$ globin chain was separated earlier by HPLC method from CKL Bart's globin. Figure 20 also shows the positions of $^{A}\gamma$T-15 which has been separated from $^{A}\gamma$ globin chain separated by HPLC method from CKL Bart's globin. $^{T}\gamma$ globin was separated from GTT hydrops but not characterized further. In the past

these tryptic peptides could not be separated from each other by any of the cation or anion chromatographies, paper electrophoresis, paper chromatography or gel filtration in various combinations. The other advantages of HPLC methods as applied to hemoglobin include the isolation of very hydrophobic peptides by using powerful organic elutropic solvents and detection of peptides with blocked amino terminus by using nondestructive methods and monitoring at a sensitive wavelength (220 nm). Due to the increased sensitivity in detection Schroeder et al. (56) were able to detect 50 picomole of a peptide without any difficulty. The reproducibility of retention times of various zones provides a good basis to compare chromatograms in order to find any aberration.

Figure 19 shows separation of 1.0 mg of aminoethylated $^{G}\gamma$ tryptic peptides by the HPLC method. Most of the tryptic peptides were recovered with an average yield of about 70% with some peptides as low as 30%. Peptide γT-13 (a cysteinyl peptide) could not be accounted for in any of the zones. It is assumed that γT-13 binds to the column and the separation conditions used may not have dislodged it from the reverse phase column. Johnson et al. (68) were able to recover cystinyl peptides of globin chains by oxidizing the sample prior to digestion and then separating the tryptic peptides on a DuPont Zorbax ODS column. Oxidation, of course, will destroy the tryptophanyl peptides.

Figure 20 shows separation of 0.5 mg of aminoethylated tryptic peptides of purified zeta chain by HPLC method using ammonium acetate-acetonitrile developer. Prior to amino acid analysis each zone was re-chromatographed by the HPLC method using phosphate-acetonitrile developer. The absence of the tryptic peptide Tyr-His and the presence

of Tyr-Arg and some of the other zeta peptides identified previously by Capp et al. (7) provided good evidence that the zeta-chain separated by newly developed HPLC from Hb Portland I or II samples does not have any gamma or beta chain contamination.

The amino acid composition of zeta tryptic peptides from purified zeta globin provides information about the peptides and a basis for comparison with alpha tryptic peptides to find out if these globin chains bear any resemblance. This is particularly true since data available from previous work has been derived from either partially purified zeta chain or from the mixture of various globin chains as shown in Table 2. From the examination of some of the tryptic peptides it appears that the zeta chain is very unique. Of the twelve tryptic peptides separated by HPLC method two zeta peptides seem to have amino acid composition identical to alpha tryptic peptides; ζL is identical to αT-10 and ζJ resembles αT-15. Two peptides, ζA and F, are similar to αT-2 and αT-5, respectively. However several tryptic peptides, viz., ζB, E, G, I, K, M and N are unique.

ζ1 is unique in having a sequence of ALA-HIS-GLY-SER-LYS, corresponding to αT7-8 (GLY-HIS-GLY-LYS-LYS). This sequence was determined by Dr. Robert Becker at Oregon State University, Corvallis. Because of its unique mobility on A-5 chromatography and because of the absence of a peptide with such a sequence in any of the known globin chains, this peptide is a very useful marker for detecting the presence or absence of zeta chain in any globin sample. The relative proportion of zeta to gamma in any sample, therefore, can be estimated by calculating the areas under the XVIII (ζ1) and XIX (γT-7) peaks as shown in Figure 11.

Table 11 shows the zeta tryptic peptides isolated from purified zeta chains of Hb Portland I ($\zeta_2\gamma_2$) of GTT hydrops and Hb Portland II ($\zeta_2\beta^?_2$) of CKL, CTY and CH have identical retention times. So far no differences in amino acid composition of tryptic peptides of zeta chains from these different Portland samples has been detected. Proudfoot et al. (67) have recently shown that ζ1(3') gene may be nonfunctional due to the presence of UAG (termination codon) sequence corresponding to amino acid number six of the polypeptide. It is, therefore, unlikely that there would be two different kinds of zeta polypeptides, unless there is another as yet unidentified active zeta locus in hydropic neonates.

The tryptic peptides ζE and G which correspond to ζ83-92 could not be detected by HPLC procedures on the C18 Lichrosorb column during tryptic peptide separation in any of the above mentioned zeta globin samples. Lauer et al. (12) have sequenced a DNA fragment from the ζ1(3') clone which corresponds to the partial amino acid sequence data of Clegg (unpublished data). Based on these findings Lauer et al. reported that the DNA sequence of the ζ1(3') fragment corresponds to the amino acid sequence of a zeta globin chain. Therefore, they proposed that the zeta chain is the product of ζ1(3') globin gene. However, since ζ2(5') rather than ζ1(3') appears to be the functional gene (67). It is proposed that the DNA sequence for 73-96 of ζ2(5') is likely to be identical to the corresponding sequence in the ζ1(3') gene. Partial amino acid sequence of tryptic peptides of zeta globin of Clegg, therefore, is very likely a product of ζ2(5') gene and not that of ζ1(3') as reported by Lauer et al.

Two tryptic peptides with the composition of ζE and ζG which

TABLE 11

Proposed ζ Globin Chain Alignment Based on Examinations of ζ Tryptic Peptides of Various Hydropic Neonates

		PREVIOUS WORK			PRESENT WORK* (ζ Globin) Retention Times (Min) of Various Peptides					
Helix	α	ζ(10)	Capp's (8) Designation of ζ Peptides	ζ (3') DNA Seq (12)	CFM	GTT	CKL	CTY	CH	Proposed ζ Alignment and Sequence
NA1	1 Val									
NA2	2 Leu									
A1	3 Ser									
A2	4 Pro									
A3	5 Ala									
A4	6 Asp									
A5	7 Lys									
A6	8 Thr	(Thr								
A7	9 Asn	Ser								
A8	10 Val	Leu	ζA		26.8	27.0	26.3	26.4	27.0	ζA
A9	11 Lys	Lys)								
A10	12 Ala	(Asn								
A11	13 Ala	Ala								
A12	14 Try	Try								
A13	15 Gly	Gly								
A14	16 Lys	Lys)								
A15	17 Val	(Ile								
A16	18 Gly	Ser								
AB1	19 Ala	Thr								
B1	20 His	Asp								
B2	21 Ala	Thr								
B3	22 Gly	Thr								
B4	23 Glu	Glu	ζB		38.0	40.0	38.7	40.1	40.1	ζB
B5	24 Tyr	Ile								
B6	25 Gly	Gly								
B7	26 Ala	Thr								
B8	27 Glu	Glu								
B9	28 Ala	Ala								
B10	29 Leu	Leu								
B11	30 Glu	Glu								
B12	31 Arg	Arg)								
B13	32 Met	(Leu								
B14	33 Phe	His								
B15	34 Leu	Leu								
B16	35 Ser	Ser								
C1	36 Phe	Phe	ζF		57.4	60.6	58.1	60.0	60.3	ζF
C2	37 Pro	Pro								
C3	38 Thr	Thr								
C4	39 Thr	Gln								
C5	40 Lys	Lys)								
C6	41 Thr									
C7	42 Tyr									
CE1	43 Phe									
CE2	44 Pro									
CE3	45 His									
CE4	46 Phe									
CE5	47 Asp	(Leu								
CE6	48 Leu	Leu								
CE7	49 Ser	Ser								
CE8	50 His	His								
CE9	51 Gly	Gly								
E1	52 Ser	Phe								
E2	53 Ala	Ala								
E3	54 Gln	His								
E4	55 Val	Val								
E5	56 Lys	Lys)								
E6	57 Gly	(Ala								Ala
E7	58 His	His								His
E8	59 Gly	Gly	ζI		7.8	8.1	7.6	8.3	8.7	Gly
E9	60 Lys	Ser								Ser
E10	61 Lys	Lys)								Lys

*Purified ζ globin chain from various hydropic neonates. Retention time (minutes) of various tryptic peptides separated by HPLC method and amino acid composition is given in Tables 7 and 9.

	PREVIOUS WORK				PRESENT WORK* (ζGlobin) Retention Times (Min) of Various Peptides					
Helix	α	ζ(10)	Capp's (8) Designation of ζ Peptides	ζ (3') DNA Seq (12)	CFM	GTT	CKL	CTY	CH	Proposed ζ Alignment and Sequence
E11	62 Val	(Val								
E12	63 Ala	Ala								
E13	64 Asp	Glu								
E14	65 Ala	Ala								
E15	66 Leu	Leu								
E16	67 Thr	Thr								
E17	68 Asn	Ser								
E18	69 Ala	Ile								
E19	70 Val	Leu								
E20	71 Ala	Gly								
EF1	72 His	Pro								
EF2	73 Val	Val		Ile						Ile
EF3	74 Asp	Asp		Asp						Asp
EF4	75 Asp	Ser		Asp						Asp
EF5	76 Met	Phe		Ile						Ile
EF6	77 Pro	Lys)		Gly		ζK	47.5	48.7	48.9	Gly
EF7	78 Asn	(Asn		Gly						Gly
EF8	79 Ala	Ala		Ala						Ala
F1	80 Leu	Val		Leu						Leu
F2	81 Ser	Gly		Ser		48.1				Ser
F3	82 Ala	Ala		Lys						Lys
F4	83 Leu	Leu		Leu	ζE					Leu
F5	84 Ser	Ser		Ser						Ser
F6	85 Asp	Glu		Glu	44.7					Glu
F7	86 Leu	Val		Leu						Leu
F8	87 His	His		His						His
F9	88 Ala	Ala		Ala						Ala
FG1	89 His	Lys)		Tyr						Tyr
FG2	90 Lys	(Ile		Ile						Ile
FG3	91 Leu	Leu	ζG	Leu	29.6					Leu
FG4	92 Arg	Arg)		Arg						Arg
FG5	93 Val			Val						Val
G1	94 Asp			Asp						Asp
G2	95 Pro			Pro						Pro
G3	96 Val			Val		ζL	41.3	41.4	41.4	Val
G4	97 Asn					40.9				Asn
G5	98 Phe									Phe
G6	99 Lys									Lys
G7	100 Leu	(Ala								
G8	101 Leu	Leu								
G9	102 Ser	Ser								
G10	103 His	His								
G11	104 Cys	Cys								
G12	105 Leu	Leu								
G13	106 Leu	Gly								
G14	107 Val	Lys)								
G15	108 Thr	(Ser								
G16	109 Leu	Leu								
G17	110 Ala	Glx								
G18	111 Ala	Ala	ζE							Incorrect alignment (see text)
G19	112 His	His								
GH1	113 Leu	Leu								
GH2	114 Pro	Tyr)								
GH3	115 Ala	(Ala								
GH4	116 Glu	Glx								
GH5	117 Phe	Phe								
H1	118 Thr	Thr								
H2	119 Pro	Ile								
H3	120 Ala	Gly								
H4	122 His	Asx								
H6	123 Ala	Ala								
H7	124 Ser	Ser								
H8	125 Leu	Leu								
H9	126 Asp	Asp								
H10	127 Lys	Lys)								
H11	128 Phe	(Phe								
H12	129 Leu	Leu								
H13	130 Ala	Ala								
H14	131 Ser	Ser								
H15	132 Val	Val								
H16	133 Ser	Ser								
H17	134 Thr	Thr								
H18	135 Val	Val								
H19	136 Leu	Leu								
H20	137 Thr	Glx								
H21	138 Ser	Ser								
HC1	139 Lys	Lys)								
HC2	140 Tyr	(Tyr	ζJ		18.2	18.1	18.0	17.9	18.4	ζJ
HC3	141 Arg	Arg)								
Other unique peptides						ζM 37.5	37.4	38.9	38.9	**

**Could not be aligned with any of the alpha-tryptic peptides.

were obtained from Hb Portland I sample of CFM hydrops and separated by A-5 chromatography are of unusual interest for a variety of reasons. Both of these tryptic peptides (ζE and G) were reported by Capp et al. also. Zeta-E is the only tryptic peptide without any lysyl, arginyl or aminoethyl cystinyl residue in its composition. Unless cleaved by a different mechanism than that of trypsin, any peptide with such a composition is likely to be a carboxy terminal peptide. Kimuzora et al. (10) have aligned ζE with αT-12. Since the nucleotide sequence of ζ1(3') of a fragment corresponding to ζ73-96 contains a peptide composition identical to ζE (ζ83-89) which in fact corresponds to a portion of αT-9, it can be argued that unless another peptide with a similar composition is present in the zeta chain, the alignment of ζE with αT-12 must be incorrect. If one were to accept the alignment of ζE with ζ83-89 because its composition fits well with a portion of DNA sequence, then some comments are required to explain how the cleavage between Tyr-Ile peptide bond (corresponding to ζ89-90 between tryptic peptide ζE and ζG) can take place. This is particularly true since trypsin is generally believed to catalyze the hydrolysis of only those peptide bonds involving the carboxyl group of lysine, arginine and aminoethyl cysteine.

The sequence of ζ1(3') 88-91 (Ala-Tyr-Ile) as reported by Lauer et al. (12) is very unique as far as the hemoglobin chains are concerned. Since the resulting split of ζG and ζE has been obtained in almost equal proportion and since no other chymotryptic-like hydrolysis was observed, it is proposed that somehow the ζ88-91 amino acid sequence of Ala-Tyr-Ile provides a site for hydrolysis to trypsin. A similar cleavage was reported (69) in Triosephosphate Isomerase (Rabbit) where

trypsin cleavage between Tyr-Ile bond was observed. The polypeptide Triosephosphate Isomerase also had a sequence of Ala-Tyr-Ile. Several polypeptides with a similar sequence of X-Tyr-Ile have been shown to have a cleavage between Tyr-Ile. Some of these peptides are listed in Table 12. Based on the information of protein sequences a sequence identical to ζ89-91 is proposed (Ile or Ala-Tyr-Ile or Leu or Gly), which is likely to be hydrolyzed between Tyr-Ile by trypsin as shown in Table 12.

It is clear that the nearest neighboring amino acid located on amino and carboxy terminus to tyrosine residue is likely to determine whether the peptide bond would be susceptible to trypsin hydrolysis or not. Based on the susceptibility of Tyr-Ile bond to trypsin the position of peptides ζE and G adjacent to each other in ζ83-92 can be explained. However it is not clear from the present work why ζE and G could be identified from the trypsin treated sample of Hb Portland I of CFM hydrops by A-5 chromatography and not from the trypsin treated purified samples of zeta chain from CKL, GTT, CTY or CH hydrops. One explanation that appears plausible is that perhaps by the HPLC separation method the yields of ζE and G peptides were very poor and hence could not be detected in CKL, GTT, CTY and CG zeta-chain samples. Another possibility is that perhaps infants with homozygous alpha-thalassemia produce two kinds of zeta chains, one containing a sequence identical to ζ83-92 (ζE+G) as shown in CFM ζ chain and DNA sequence of ζ1(3') and another with some different sequence. Further work is suggested to clarify this point in hydropic neonates with homozygous alpha-thalassemia.

TABLE 12

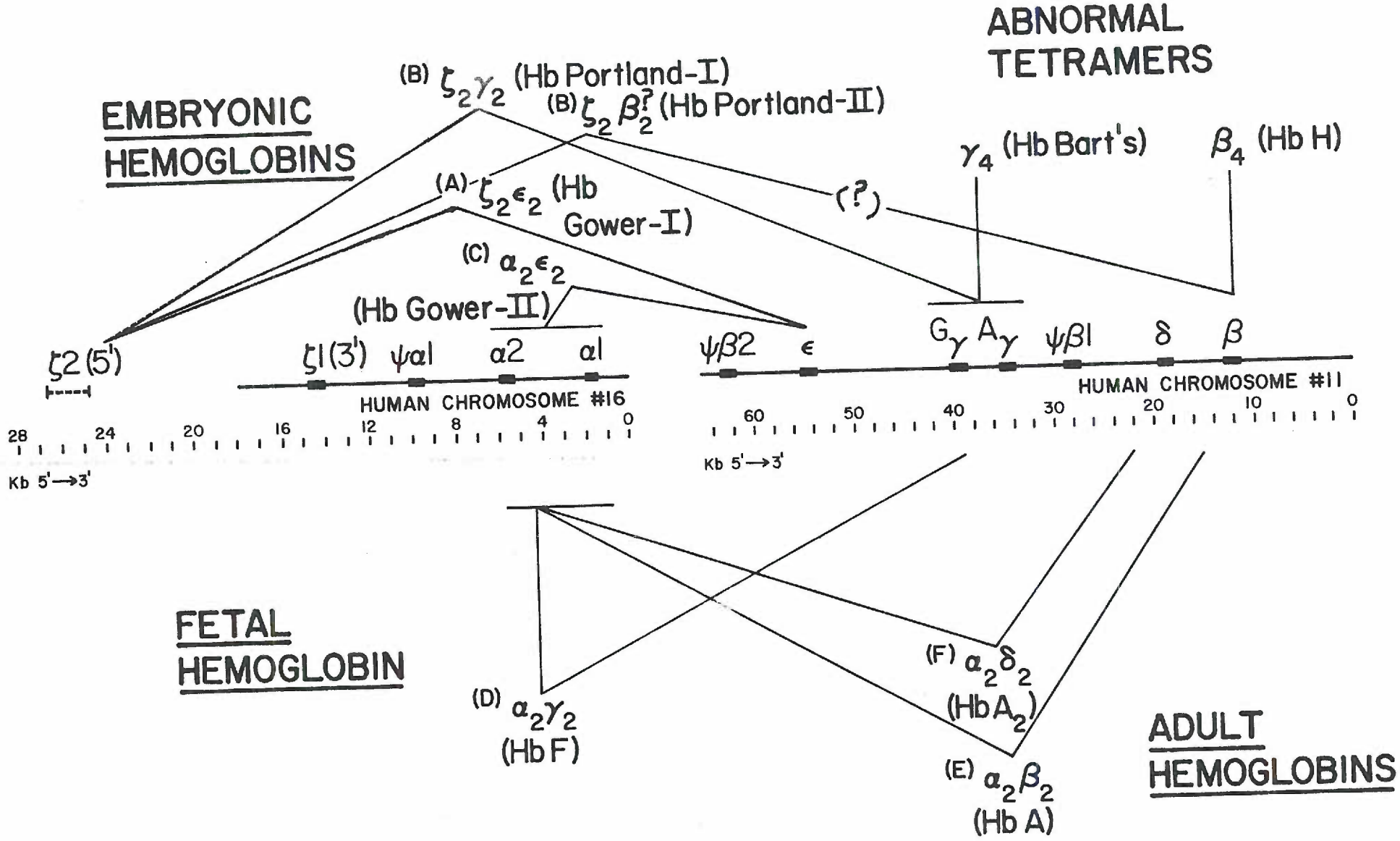
(A) Trypsin Sensitive X-TYR$\downarrow$Y Peptide Bonds

Amino Acid Sequence and Position	Protein, Source and Reference
ALA-TYR$\downarrow$ILE 89 90 91	ζ chain (Hb Portland I); present work and data from DNA sequence of ζ (3') gene (12)
ALA-TYR-ILE 47 48 49	Triosephate isomerase, rabbit (69)
ALA-TYR-LEU 47 48 49	Triosephate isomerase, coelacanth (69)
ILE-TYR-LEU 47 48 49	Triosephate isomerase, chicken (69)
ALA-TYR-LEU 96 97 98	Cytochrome 'C', Lamprey (70)
ALA-TYR-ILE 273 274 275	Pepsinogen, pig (71)
ALA-TYR-GLY 235 236 237	Pancreatic deoxy ribonuclease 'A', bovine (72)
ALA/ILE-TYR-ILE/LEU/GLY (X -TYR- Y)	The cleavage between TYR$\downarrow$Y will occur only when X and Y are any of the listed amino acids.

(B) Trypsin Insensitive X-TYR-Y Peptide Bonds

ALA-TYR-ASP 207 208 209	Pancreatic deoxy ribonuclease 'A', bovine (72)
SER-TYR-ILE 23 24 25	Pancreatic deoxy ribonuclease 'A', bovine (72)
VAL-TYR-LEU 66 67 68	Cytochrome 'C', Lamprey (70)
GLU-TYR-LEU 66 67 68	Cytochrome 'C', human (73)
LYS-TYR-ILE 73 74 75	Cytochrome 'C', Lamprey (70)

Figure 23. Diagram showing the location and association of various globin chains to form human embryonic, fetal and adult hemoglobins. The evidence for the existance of Hb Portland II is given in this dissertation. Hb Portland II is believed to be an embryonic hemoglobin. ζ_1(3') has recently been shown to contain UAG terminator at the codon for amino acid 6, hence is believed to be incompatible with normal gene function (67). Evidence for ζ_2(5') to be a functional gene has been shown recently (62).


ABNORMAL TETRAMERS
EMBRYONIC HEMOGLOBINS
(B) $\zeta_2\gamma_2$ (Hb Portland-I)
(B) $\zeta_2\beta_2^?$ (Hb Portland-II)
γ_4 (Hb Bart's)
β_4 (Hb H)
(A) $\zeta_2\epsilon_2$ (Hb Gower-I)
(?)
(C) $\alpha_2\epsilon_2$ (Hb Gower-II)
$\zeta 2$ (5')
$\zeta 1$ (3') $\psi\alpha 1$ $\alpha 2$ $\alpha 1$
HUMAN CHROMOSOME #16
28 24 20 16 12 8 4 0
Kb 5'→3'
$\psi\beta 2$ ϵ G_γ A_γ $\psi\beta 1$ δ β
HUMAN CHROMOSOME #11
60 50 40 30 20 10 0
Kb 5'→3'
FETAL HEMOGLOBIN
(D) $\alpha_2\gamma_2$ (Hb F)
(F) $\alpha_2\delta_2$ ($Hb A_2$)
(E) $\alpha_2\beta_2$ (Hb A)
ADULT HEMOGLOBINS

SUMMARY

Protocols for the isolation and purification of Hb Portland I ($\zeta_2\gamma_2$), Hb Portland II ($\zeta_2\beta^?_2$), Hb Bart's (γ_4) and various hemoglobin components from the red cells of neonates with homozygous alpha-thalassemia have been developed. The methods which have been employed to separate and identify various hemoglobins and their constituent globin chains include starch gel electrophoresis, analytical and preparative isoelectricfocusing, cellulose acetate electrophoresis, Triton X100 polyacrylamide gel electrophoresis, countercurrent distribution method, Aminex 5 cation exchange peptide chromatography, and analytical and preparative reverse phase high performance liquid chromatography.

Four hemoglobin components have been separated by starch gel electrophoresis from the hemolyzates of blood from some hydropic neonates. Components I, II, and III are identified as Hb Bart's, Hb Portland I, and Hb Portland II, respectively. Component IV is postulated to be Hb Portland III ($\zeta_2\delta_2$) and like component III (Hb Portland II) is present in some but not all hydropic neonates.

The existence of Hb Gower I ($\zeta_2\varepsilon_2$), Hb Portland I ($\zeta_2\gamma_2$), Hb Portland II ($\zeta_2\beta^?_2$) and the postulated Hb Portland III ($\zeta_2\delta_2$) indicates that the zeta chain is alpha-like since alpha-globin also forms similar hemoglobin tetramers, viz. Hb Gower II ($\alpha_2\varepsilon_2$), Hb F ($\alpha_2\gamma_2$), Hb A ($\alpha_2\beta_2$) and Hb A_2 ($\alpha_2\delta_2$). Hydropic neonates with homozygous alpha-thalassemia form zeta-containing hemoglobin tetramers to compensate for the lack of alpha-containing tetramers. It is postulated that all of these hemoglobins are physiologically functional.

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ADDENDUM

Since submitting this thesis in September, 1981, two publications describing the amino acid sequence of the zeta chain have appeared. They are "Human Embryonic Hemoglobin: The Primary Structure of the ζ-Chain" by H. Aschauer, Sanguausermsri, T., and Braunitzer, G. in Hoppe-Seyler's Zeitschrift fur Physiologische Chemie Vol. 362, No. 8, 1159-1162, 1981, and "Embryonic Hemoglobin: Sequence of the ε and ζ Chains" by J. B. Clegg in Texas Reports on Biology and Medicine, 40, 23-28, 1980-81.

Additional work was carried out to further characterize Hb Portland II and its $\beta^?$ chain. The material present in the RT 15 zone was compared with known samples of α, β, $^A\gamma$, $^G\gamma$, and ζ chains by globin-chain electrophoresis on polyacrylamide gel using the method of Alter et al. (45). The RT 15 zone was found to have a single component with a mobility identical to the normal β chain. A sample of hemolysate from CKL hydrops was shown to have a zone which co-chromatographs with the normal β chain when separated by HPLC using the long duration program (GCSAL). Because this hemolysate contained only Hb Bart's (γ_4), Hb Portland I ($\zeta_2\gamma_2$) and Portland II ($\zeta_2\beta^?_2$) but no Hb H (β_4), it is concluded that the $\beta^?$ chain of Hb Portland II has the chromatographic properties of the normal β^A chain.

Except for the presence of less than one residue of isoleucine in the analysis of the amino acid composition of $\beta^?$ of RT 15 from CKL (Table IV) which can be attributed to a small contamination by γ chain, all of the studies of the $\beta^?$ chain of Hb Portland II indicate it is identical to the normal β^A chain.