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NONUNIFORM BIOSYNTHESIS OF MULTIPLE HEMOGLOBINS IN THE ADULT GUINEA PIG

by

WIN LIN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1977

Dedicated

with love, admiration and gratitude

to

my parents

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

2/11/77
date

Abraham Mazur
Chairman of Examining Committee

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date

Aaron Lukton
Executive Officer

ABSTRACT

Nonuniform Biosynthesis of Multiple Hemoglobins in the Adult Guinea Pig

by

Win Lin

Adviser: Professor Abraham Mazur

Two hemoglobin components were found in red cells of guinea pig by the methods of disc gel and isoelectric focusing gel electrophoresis. The same two hemoglobin components were also revealed in marrow cells on the isoelectric focusing gel. The major hemoglobin component in red cells was the minor one in marrow cells. By in vivo ^{59}Fe labeling studies, it was proven that hemoglobin 2 is associated with the youngest cells and hemoglobin 1 associates with the older cells. The two hemoglobins are nonuniformly synthesized in the adult guinea pig during their development. This conclusion was further confirmed by the method of centrifugation in a dextran density gradient, in which the multiple hemoglobins in marrow cells can be separated according to their relative stages of maturation. The results of studies of two guinea pig hemoglobins suggest that the phenomenon first observed in the rat may be a general one among mammalian multiple hemoglobins. The finding of nonuniform biosynthesis of two multiple hemoglobins in the guinea pig poses the question of assembly of the correct α and β chains for each of these hemoglobins at the proper stage of erythroid cell maturation. If all or some of the α or β chains were identical, the complexity of regulating the transcription of these chains would be lessened. Also, it is possible that early chains might serve as precursors for later chains. To resolve this question, analyses of hemoglobins 1 and 2 have been performed after their separation and purification. By the criteria of amino acid analysis, peptide mapping and amino acid sequencing by homology the two α chains as well as the two β chains are considered different. Therefore it is suggested that four different structural genes code for the four different polypeptide chains. This rules out the possibility that early chains might serve as precursors for later chains and makes the regulatory mechanism of gene transcription more complicated.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Multiple hemoglobins have been reported in adult mammalian red cells. e. g., human (Schneck et al., 1961), rat (Stein et al., 1971) and mouse (Popp, 1965). In the rat, it has been demonstrated that six hemoglobins of the adult red cell are present in marrow erythroid cells and these six hemoglobins are nonuniformly synthesized in the erythroid cells during cell maturation. Rat hemoglobin 5 is synthesized most actively in the earliest erythroid cells whereas hemoglobin 4 (the major hemoglobin of the red cell) is synthesized most actively in the latest erythroid cells. The recent data of Yeh (Weiser et al., 1976) has confirmed the reality of nonuniform biosynthesis of rat hemoglobins in marrow erythroid cells by identification of different relative distribution of multiple hemoglobins in erythroid marrow cells separated according to their relative stages of maturation.

The control mechanisms involved in nonuniform hemoglobin synthesis in many species has become a subject of great interest in recent years. Most of the work was done by analysis of hemoglobin polypeptide chains of various mammalian species to suggest genetic mechanisms that cause chemical heterogeneity of these proteins. Thus far structural variability of mammalian hemoglobins has been studied primarily in humans (Huisman et al., 1971) in which the occurrence of hemoglobin heterogeneity was found to be due to structural differences in β and δ chains, accounting for hemoglobin A and A_2 when

they are combined with a structurally identical α chain. In the horse, two distinct hemoglobin components were reported and evidence has been found that the structural differences was in the α chains (Clegg, 1974). Chemical studies of the six purified rat hemoglobins, performed by Stein (Stein et al., 1971) demonstrated that the heterogeneity was due to differences in the primary structure of the globin chains and not to induced chemical modifications, artifacts of isolation or to differences in aggregation. Several mechanisms were suggested for controlling the variations in amino acid sequences of some α or β chains of mammalian hemoglobins as following:

1) the replacement of one amino acid by another;

2) a nonhomologous crossing-over between corresponding points of the genes for different chains;

3) deletion mutations resulting in the loss of one or more amino acids in a given polypeptide chain;

4) the existence of different multiple templates for the specific chain;

5) the variable translation of a unique template correlated with degeneracy of the genetic code.

In order to determine whether the non-uniform biosynthesis of multiple hemoglobins in the rat is a general phenomenon in other species, the adult laboratory guinea pig and mouse were studied. My approach dealing with this problem was first to use the technique of isoelectric focusing electrophoresis to resolve both red blood cell and bone marrow

cell hemoglobins on polyacrylamide gels. Bone marrow cells contain mostly immature erythroid cells and red blood cells contain the fully matured erythroid cell. One indication of non-uniform hemoglobin synthesis in erythroid cells is the finding that the percentage distribution in hemoglobin content of both kinds of cells is distinctively different, i.e., two hemoglobin patterns on the gel are different. The conclusive evidences for this concept will then be provided by *in vivo* ^{59}Fe-labeling studies and by identification of different relative distribution of multiple hemoglobins in erythroid cells separated according to their relative stages of development.

To approach an understanding of the control mechanisms involved in hemoglobin synthesis was another goal of this dissertation. In an attempt to achieve this, the following approaches were applied:

a) a preparative adaptation of isoelectric focusing electrophoresis in polyacrylamide gels to separate and isolate each of the multiple hemoglobins in a pure state;

b) separation of hemoglobins into their polypeptide chains;

c) comparison of the total amino acid compositions of each α and β chain;

d) comparison of the two-dimensional peptide maps of the tryptic digests of each α and β chain;

e) comparison of the amino acid content of α and β

tryptic peptides and their sequence assigned by homology with other mammalian hemoglobins. Depending upon the nature of the amino acid sequence differences in the α and β chains, one would be able to speculate about genetic mechanisms that cause chemical heterogeneity among the hemoglobins.

EXPERIMENTAL

Materials

Female Albino guinea pigs (New Zealand White) were purchased from Marland Breed Farm, New Jersey. All the chemicals used in this thesis were Fisher Scientific reagent grade unless stated otherwise. Dextran (Clinical grade, average Mw 83,300) and 2-Mercaptoethanol were produced by Sigma Chemical Company. Bovine serum albumin was from Armour Laboratory, Kankakee, Ill. Ampholines (pH 6-8) were from LKB Instrument Company. N,N,N', N'-tetramethylethylenediamine (TEMED), acrylamide and N,N'-methylene bis-acrylamide for the isoelectric focusing experiments were purchased from Eastman. DEAE-cellulose and AG 501-X8(D) were supplied by Bio-Rad. Sephadex G-25 was from Pharmacia Fine Chemicals. CM-52 cellulose was obtained from Whatman Company. Trypsin treated with TPCK was purchased from Worthington Biochemicals. Ninhydrin was from Pierce Chemicals as was the 4N Methane sulfonic acid containing 0.2% 3-(2-aminoethyl)Indole. All the buffers used for amino acid analysis were bought from Beckman.

Methods

Preparation of Hemoglobin from Red Blood cells or Bone Marrow Cells

Blood was collected from the heart or central artery of anaesthetized guinea pigs in heparin-isotonic 0.9% NaCl solution and the red blood cells were washed three times with isotonic 0.9% NaCl solution by centrifugation. The packed

cells were then lysed at room temperature by addition of four volumes of a solution of 10^{-4}M EDTA (pH 7.4) and the solution was saturated with carbon monoxide. One-tenth of its volume of 9% NaCl solution was added and the cell stroma was removed by centrifugation at 20,000 X g for 20 min. The hemoglobin solution was dialyzed overnight in the cold, with stirring, against a large volume of 0.005M Tris-HCl, pH 8.6. After dialysis, the hemoglobin solution was then placed onto a DEAE-cellulose column equilibrated with 0.005M Tris-HCl, pH 8.6. The column was washed with 50 ml of the same buffer to remove any impurities. The purified hemoglobin was collected by eluting with 0.1M KPO_4-0.1M NaCl (pH 7.0). The solution was then dialyzed in the cold against 0.005M Tris-HCl (pH 8.6) in the presence of carbon monoxide. The preparation of hemoglobin from bone marrow cells was the same.

Preparation of Bone Marrow Cells

Normal guinea pigs weighing in the range of 200-250 gms were anaesthetized by intramuscular injection of Nembutal Sodium (5 mg/100 gm body weight) and were also injected intravenously with 0.1 ml heparin (1000 U/ml). After a few minutes, the four long bones from the hind legs were quickly removed. Both ends of each bone were cut with scissors and punctured with a NO. 20 hypodermic needle. The marrow cells were forced out in a single plug with a 2 ml syringe, containing saline solution. The marrow cells were washed several times with saline solution, for preparation of hemoglobin, or with the

standard medium, for separating cells of different ages. The cells were dispersed during washing by repeated pipetting, and then passed through graded wire sieves. After each washing, the cells were centrifuged at 5^{o}c for 20 min at 1,000 x g. At this point the bone marrow cells were ready for preparation of total hemoglobin or for separation of the cells by density gradient centrifugation.

Praparation of Dextran Gradients

A stock solution of 30% (w/v) dextran was prepared by dissolving the dextran in a standard medium [136.9 mM NaCl; 5.36 mM KCl; 1.08 mM Na_2HPO_4; 1.10 mM KH_2PO_4; 0.63 mM $MgSO_4 \cdot 7\ H_2O$; 6.11 mM glucose and 0.5% bovine serum albumin (w/v)] and then centrifuging the solution at low speed to remove the bubbles. Twenty percent (w/v) dextran solution was prepared from the 30% stock solution by diluting with standard medium. These concentrations were then used as the limits of the density gradient. Linear gradients were formed at low drive speed by use of a Beckman Density Gradient Former supplied with a Triple Mixing Chamber. The gradient was prepared by the action of two cam-operated pushers against a pair of 50 ml lubricated glass syringes, one containing 30% dextran solution, the other containing 20% dextran solution. Gradients were filled to within 1 cm from the top of 1 x 3 inch centrifuge tubes. (Schulman, 1967)

The Fractionation of Bone Marrow Cells in Dextran Density Gradients

Packed bone marrow cells suspended in 20% dextran solu-

tion were carefully layered on top of the gradient and centrifuged at 13,200 x g for 35 min in the Swinging-Bucket SW 25.1 rotor of a Beckman Model L Ultracentrifuge. Five different bone marrow cell fractions were collected manually from the top by use of a pipette. These fractions were then washed four times with 0.9% NaCl solution, frozen and then lyzed by adding 10^{-4}M EDTA (pH 7.0) solution. The hemoglobin concentration of the top fraction usually too dilute for gel electrophoresis required further concentration in the cold in narrow dialysis bags wrapped in Sephadex G-200 powder for serval hours.

Analytical Polyacrylamide Gel Electrophoresis

Analytical disc gel electrophoresis was performed in glass tubes (0.7 x 10 cm) with 0.05 M Tris-glycine, pH 8.6, as the buffer and a current of 4 ma per tube for 1 hour. Bromphenol blue was used as a tracking dye. (Davis, 1964)

Analytical isoelectric focusing electrophoresis was performed in polyacrylamide gels containing a mixture of ampholytes which established a pH gradient from 6.0 to 8.0. Each gel tube, 0.7 x 10 cm, contained 0.5 mg of carboxyhemoglobin previously dialyzed against 0.005 m Tris-HCl buffer, pH 8.6. Electrophoresis was performed at 50 volts for 3-4 days in the cold room (Dale et al., 1968; Wrigley, 1968). Unstained gels were subjected to densitometric analysis.

Preparative Isoelectric Focusing Separation of Guinea Pig Hemoglobins

Guinea pig hemoglobins on a preparative scale (40 mg) were separated into its 2 components as previously described

except that electrophoresis was performed at 75 volts for 72 hours using glass tubes 1 x 13 cm in size. After completion of electrophoresis, the gels were removed from the tubes, sliced as close to the bands as possible and stored at -20^{0}C.

In Vivo Study with ^{59}Fe

Normal guinea pigs were anaesthetized by intramuscular injection with sodium pentobarbital (0.1 ml) and also intravenously with 0.75 ml radioactive ferric citrate (8 x 10^{6} cpm/ml). Small quantities of blood (0.3-0.4 ml) were removed by cardiac puncture at various time intervals following injection. The hemoglobins from the washed red cells were separated on preparative isoelectric focusing gels and extracted. Their specific activities (cpm/mg Hb) were determined in a well-type crystal scintillation counter (Nuclear Chicago Company).

Extraction of Hemoglobin Fractions from IEF Gel Slices

Gel slices were homogenized in a Virtis Homogenizer with 0.05 M Potassium phosphate-0.1 M NaCl, pH 8.6 extraction buffer. After centrifugation at 3,000 rpm, precipitated acrylamide gel was discarded. The supernatant hemoglobin solution was used for radioactivity estimation.

For preparation of globin, the hemoglobin solution was further purified by exhaustive dialysis against large volumes of 0.005 M Tris-HCl, pH 8.6 buffer and adsorbed onto a DEAE-cellulose column as previously described. The eluted hemoglobin solution was then desalted by passing through a Sephadex G-25 column, 2.5 x 30 cm, equilibrated with 0.01 M KPO_4, pH 8.0

buffer and finally dialyzed against large volumes of 0.005 M Tris-HCl, pH 8.6 buffer in the cold.

Preparation of Globin

Hemoglobin was converted to globin by treatment with an excess of 2% HCl-acetone containing 0.05 M 2-mercaptoethanol at -20°C. Care was taken to remove all acid by washing the precipitate 4 times with cold (-20°C) acetone and once with ether. Finally, the ether-washed precipitate was dried in the dessicator under vacuum. 2-mercaptoethanol was used throughout the entire preparation to prevent oxidation.

Separation of Globin into α and β Chains

The two guinea pig globins were separated into their α and β chains by use of a urea-phosphate gradient (Clegg et al., 1966). The dry globin (40 mg) was dissolved in chromatography starting buffer (8 M urea made 0.05 M with 2-mercaptoethanol, 0.005 M in Na_2HPO_4, and adjusted to pH 6.7 with H_3PO_4) to a concentration of 4-10 mg/ml. The globin solution was dialyzed against 3 changes of 50-fold excess of starting buffer for 3 hours at room temperature before it was applied to a 1 x 40 cm CM-cellulose column. After unbound material was removed, the globin chains were eluted at a flow-rate of 1 ml/1½ min by means of a linear Na^+ ion gradient formed by mixing 150 ml of starting buffer with 150 ml of 8 M urea made 0.05 M in 2-mercaptoethanol, 0.03 M in Na_2HPO_4 and adjusted to pH 6.7 with H_3PO_4. Three ml fractions were collected at room temperature. 8 M urea used for these solutions was filtered through filter

paper, and then deionized through a 2 x 30 cm columm of AG 501-X8(D) mixed resin bed (20-50 mesh) at a rapid rate before use. Conductivity of the urea solution should be less then 10U mho^{-1}.

The fractions containing polypeptide chains were pooled and desalted by passing through a 3 x 120 cm Sephadex G-25 column equilibrated with 0.5 % formic acid, before lyophylizing. Formic acid was then removed from the protein by redissolving the protein in water and lyophilizing once again.

Amino Acid Analysis of Individual Chains

A sample of each individual chain was hydrolyzed with 6N HCl at 110°C for 24, 48, 72 hours in evacuated, sealed ampoules. Each hydrolysate was then freed of HCl and analyzed for amino acid on a Beckman Model 118 Analyzer.

The cysteine residues were determined as cysteic acid after performic acid oxidation (Hirs, 1967)

Tryptophan was determined by hydrolyzing with 4N methane sulfonic acid containing 0.2% 3-(2-aminoethyl) Indole (Liu, 1971).

Labile amino acids, serine and threonine, were determined by extrapolating values obtained from 24, 48 and 72-hour hydrolyzates to zero time.

Tryptic Digestion of Globin

The globin was dissolved in water (10 mg of protein/ 1 ml of H_2O) and made 1% with respect to NH_4HCO_3. The solution was brought to pH 9.0 with NH_4OH. Trypsin (1% solution in 0.001N HCl) was added to give enzyme/substrate ratio of

1/100. Digestion was carried out overnight at room temperature and terminated by adjusting the pH to 4.5 with acetic acid. After centrifugation, the supernatant, containing the soluble tryptic peptides, was lyophilized twice from water.

Separation of the Tryptic Peptides

A sample of soluble tryptic peptides (equivalent to 1-2 mg of digested globin) was applied to a sheet of Whatman 3 MM paper (18' x 23'). Descending chromatography was carried out at room temperature for 16 hours. The buffer system employed was n-butanol-acetic acid-water (4:1:5 by volume) (Katz et al., 1959).

After drying in air, the paper was suspended in a "Varsol"-cooled tank (Savant Instruments, N.Y.) with the line of sample nearest the anode. High voltage electrophoresis was then performed in a pyridine-acetic acid-water buffer (10:0.4:90 by volume) (pH 6.4) at 2000 volts for 2¼ hours (Ingram, 1958).

Stains for Peptide Maps

Peptides were located by staining lightly with ninhydrin or with reagents for specific amino acids.

The peptide maps were developed by spraying the dried chromatograms with 0.05% ninhydrin in 95% alcohol and heating at 60°C for 20 min.

Pauly reagent staining for histidine and tyrosine was done with 0.1% diazonium salt of sulfanilic acid in 10% Na_2CO_3. The stain was developed at room temperature (Dhounlt, 1974).

Ehrlich reagent staining for tryptophan was done with

1% p-dimethylaminobenzaldehyde in acetone-HCl (90:10) and was developed at room temperature (Ehrlich, 1967).

Elution of Peptides from Peptide Maps

Four peptide maps were prepared and sprayed lightly with ninhydrin. Spots were developed by heating at 60°C before being circled and cut out from the chromatography paper. Peptides were stored in glass vials at -20°C until they were eluted. The peptides were then eluted with distilled water, lyophilized, redissolved in 6N HCl, and hydrolyzed in vacuum at 110°C for 24 hours. The hydrolysates from each peptide were analyzed using a Beckmen Model 118 amino acid analyzer.

RESULTS

Multiple Hemoglobins in Guinea Pig Red Blood Cells and Bone Marrow Cells

Analytical polyacrylamide disc gel electrophoresis at pH 8.6, as well as analytical isoelectric focusing in polyacrylamide gels along a pH gradient from 6.0 to 8.0, yielded two hemoglobin bands from hemoglobin solutions of guinea pig red blood cells (Figure 1). The method of isoelectric focusing produced sharper separation of the two bands than did disc gel electrophoresis. The band nearest the cathode was the major band and was named hemoglobin 1 in this thesis, and the minor band was named hemoglobin 2. The relative distribution of these two bands as well as their pI value are shown in Table I.

Analytical isoelectric focusing separation of hemoglobins prepared from pooled guinea pig bone marrow also revealed the same two hemoglobins with respect to their isoelectric points. Their relative distribution was reversed (Figure 2); the major hemoglobin of the red cell, hemoglobin 1 (pI 7.1) was present to the extent of 72.8% of the total whereas in total bone marrow, hemoglobin 2 (pI 6.8) constituted 85.5% of the total.

Multiple Hemoglobin Distribution in Red Cells and Marrow of the Mouse

Pooled red cells and pooled total marrow cells of several mice were washed with saline and their respective total hemoglobins extracted for analysis by analytical isoelectric

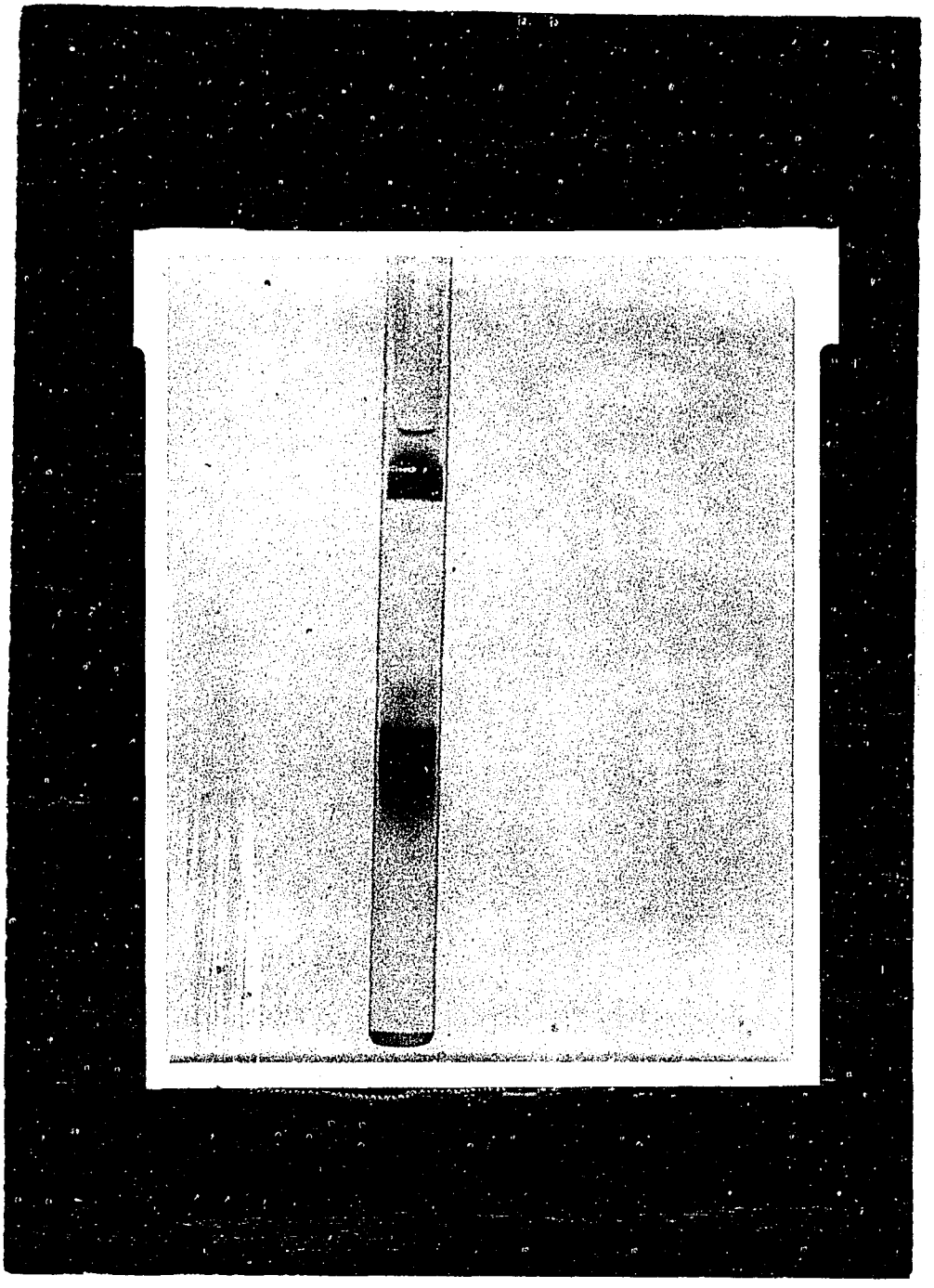


FIGURE 1

Analytical polyacrylamide gel electrophoresis of guinea pig red cell hemoglobins (pH 8.6).

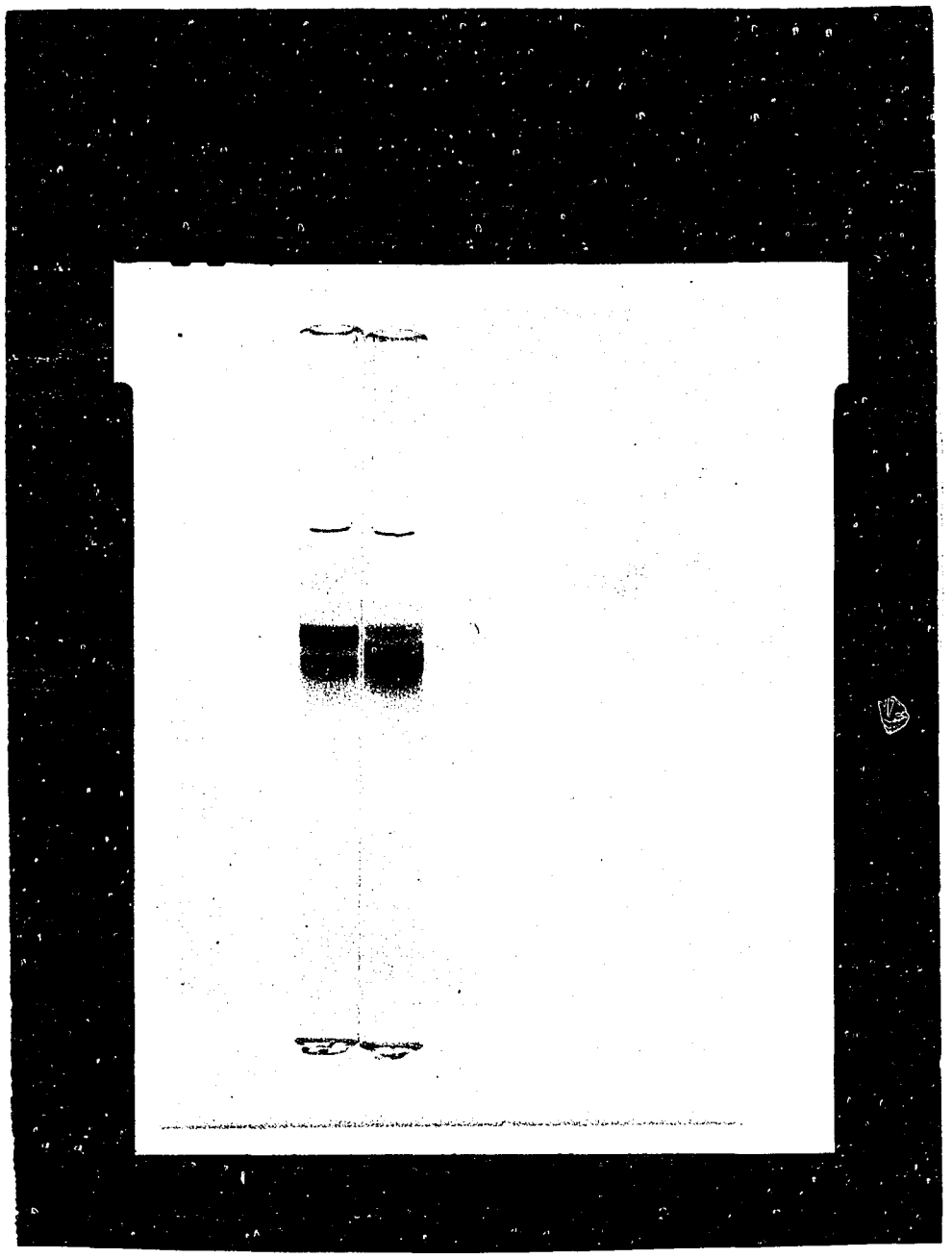


FIGURE 2

Analytical isoelectric focusing separation of guinea pig hemoglobins prepared from red cells (left) and bone marrow (right).

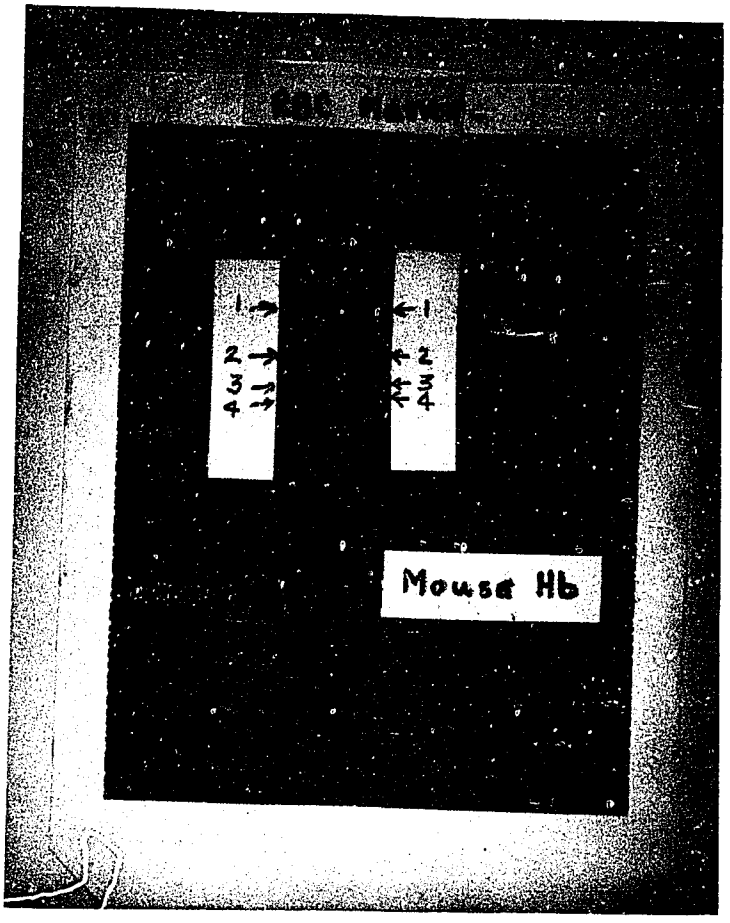


FIGURE 3

Analytical isoelectric focusing separation of mouse hemoglobins prepared from red cells (left) and bone marrow (right).

TABLE I

DISTRIBUTION OF TWO HEMOGLOBINS IN GUINEA PIG MARROW AND RED CELLS

Hemoglobins were separated by isoelectric focusing electrophoresis on polyacrylamide gels and their distribution determined from scanning optical density readings. Isoelectric points were determined by pH measurements of gel eluates.

Hemoglobin	Isoelectric point	Red cells (% distribution)	Marrow
1	7.1	72.8	14.5
2	6.8	27.2	85.5

TABLE II

DISTRIBUTION OF HEMOGLOBINS IN MOUSE MARROW AND RED CELLS

Hemoglobin fraction	Expt. 1		Expt. 2		Expt. 3	
	RBC	Marrow	RBC	Marrow	RBC	Marrow
1	12.7	8.5	5.4	14.5	3.7	4.3
2	59.6	35.3	70.3	25.7	78.1	5.8
3+4	27.7	56.2	24.3	59.8	18.2	89.9

focusing electrophoresis and optical density scanning of the gels. The results are shown in Figure 3 and Table II. Although hemoglobins 3 and 4 are not well resolved, it is obvious that the relative distribution of these hemoglobins in red cells and marrow cells are completely different, resembling the situation in the guinea pig.

In Vivo Study with ^{59}Fe in Guinea Pig

The marked difference in distribution of the two hemoglobins in total bone marrow cells as compared with the red blood cell suggested that these two hemoglobins are not made to the same extent at each stage of maturation of the erythroid cell. The incorporation of isotopic precursor into the two hemoglobins was studied. If all erythroid cells were to synthesize each of the two hemoblogins one would expect to see in the circulating red cells at all times two hemoblobins of equal specific activities. The result of this experiment is shown in Table III which illustrates the specific radioactivity ratios of hemoglobin 2 to hemoglobin 1 in circulating red cells following in vivo labeling of marrow erythroid cells with ^{59}Fe. It can be seen that at short time intervals after ^{59}Fe injection hemoglobin 2 has a low specific radioactivity. With time, the specific radioactivity of hemoglobin 2 increased and reached a value equal to that of hemoglobin 1 by 14 days. From this result one may propose that erythroid cells, at different stages of their differentiation, synthesize different hemoglobins, and at different rates. In this case hemoglobin 1 is mostly synthesized in the older erythroid cells of the marrow whereas hemoglobin 2 is synthesized mostly in the young erythroid cells.

TABLE III

RELATIVE SPECIFIC RADIOACTIVITIES OF GUINEA PIG HEMOGLOBIN IN CIRCULATING RED CELLS FOLLOWING IN VIVO LABELING OF MARROW ERYTHROID CELLS WITH ^{59}Fe

Days after ^{59}Fe	Ratio of specific radioactivities Hb 2:Hb 1		
	Experiment 1	Experiment 2	Average
0.3	0.44	0.50	0.47
1	0.65	0.57	0.61
4	0.73	0.60	0.67
8	0.82	0.65	0.74
14	0.91	1.12	1.01

The Fractionation of Guinea Pig Bone Marrow Cells in Dextran Density Gradients.

"Young" and "old" erythroid cells can be separated by density gradient centrifugation because of their differences in density. After centrifugation, the most dense cells (the old cells) would settle to the bottom of the gradient while the less dense cells(the young cells) would remain at the top.

Five fractions were collected for analyses, namely (starting at the top), I, II, III, IV and V, and their hemoglobin components were examined by isoelectric focusing gel electrophoresis. The result is shown in Figure 4. Fraction I which contains the youngest erythroid cells contains mostly hemoglobin 2. With cell maturation, hemoglobin 1 increases in relative amounts.

To further prove that density gradient centrifugation separates marrow cells of different stages of maturation, the bone marrow cells were pooled after two hours ^{59}Fe injection in vivo. The specific radioactivity of total hemoglobin in each fraction collected from the dextran density gradient was determined. The results are shown in Table IV which illustrates the specific radioactivities of the total hemoglobin extracted from cells in each fraction, indicating a relative separation based on density; the least dense cells contained the highest specific radioactivity and are assumed to be the "youngest" erythroid cells. Separation of the hemoglobins in each fraction by isoelectric focusing electrophoresis led to the finding

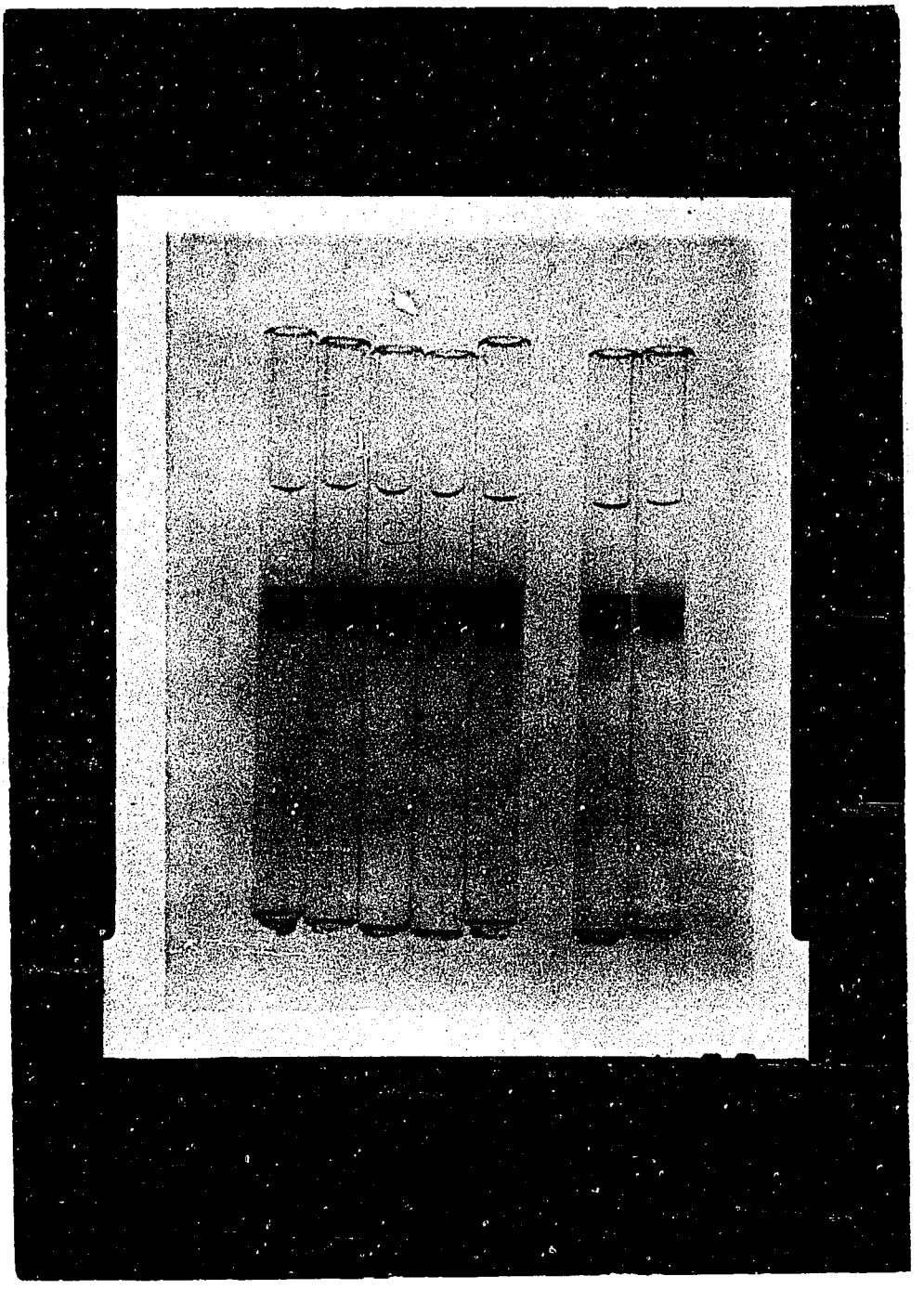


FIGURE 4

Analytical isoelectric focusing separation of guinea pig hemoglobins from marrow cells. From left to right, Fraction I (top fraction collected from dextran density gradient), II, III, IV and V (bottom fraction collected from dextran density gradient), total marrow, and total red blood cells.

TABLE IV

DISTRIBUTION OF MULTIPLE HEMOGLOBINS IN GUINEA PIG BONE MARROW CELLS SEPARATED BY CENTRIFUGATION IN A DEXTRAN DENSITY GRADIENT

Animals were injected with ^{59}Fe 2 hours prior to removal of marrow in order to identify cells at different stages of maturation.

Cells	Distribution %		Ratio	Hb specific
	Hemoglobin 1	Hemoglobin 2	Hb 1:Hb 2	radioactivity
				cpm/mg Hb
Total red blood cells	71.3	28.7	2.48	
Total marrow	25.1	74.9	0.34	
Fractions:				
(I) Top	7.5	92.5	0.08	1435
(II)	28.2	71.8	0.39	1072
(III)	32.9	67.1	0.49	852
(IV)	44.1	55.9	0.79	561
(V) Bottom	58.3	41.7	1.40	438

that the minor hemoglobin of circulating red cells, hemoglobin 2, was associated mostly with the youngest erythroid cells whereas the major hemoglobin of red cells, hemoglobin 1, is present to the greatest extent in the most dense, and therefore the "oldest" erythroid cells.

Amino Acid Composition of Guinea Pig Red Cell Hemoglobins

From the previous results, if was proved that hemoglobin 1 and hemoglobin 2 were synthesized at different stages of their differentiation. Amino Acid analyses were then performed on each of the two hemoglobin fractions in order to explore differences in chemical properties. The values in Table V for all amino acids in each of the two hemoglobins represent values obtained from 24, 48 and 72 hours hydrolysate. The number of residues for each hemoglobin tetramer was taken as 574. Arginine, isoleucine and valine with bulky side groups are released more slowly than the other amino acids and their values represent an average of the "72 hour" determinations. The values for threonine, serine and tyrosine are determined by extrapolation back to zero time in order to correct for their partial destruction during acid hydrolysis. Tryptophan and cysteine which are completely destroyed during acid hydrolysis are determined by the methods of Lui (Lui, 1971) and Hirs (Hirs, 1967) respectively. Cystine yields cysteic acid after performic acid oxidation and hydrolysis. Other amino acids, not detected by acid hydrolysis, are asparagine and glutamine. These two amino acids are deamidated and recovered as aspartic and glutamic acids, respectively. Methionine is also difficult to recover

TABLE V

AMINO ACID COMPOSITION OF GUINEA PIG RED CELL HEMOGLOBINS[a]

Amino Acid	Hemoglobin	
	I	II
Tryptophan	3.9[b]	4.0[b]
Lysine	47.3 (0.7)	45.2 (0.1)
Histidine	41.5 (1.0)	42.5 (0.8)
Arginine	11.3 (0.4)	12.0 (0.1)
Aspartic	49.8 (0.5)	52.3 (0.8)
Threonine	40.3[c]	42.8[c]
Serine	40.7[c]	40.2[c]
Glutamic	31.1 (0.4)	30.8 (0.1)
Proline	15.6 (1.1)	18.7 (0.5)
Glycine	37.5 (0.8)	39.0 (0.4)
Alanine	74.9 (0.8)	73.6 (0.7)
Cysteine	2.9[d]	3.9[d]
Valine	45.3[e]	47.4[e]
Methionine	7.7[d]	5.3[d]
Isoleucine	17.4[e]	17.3[e]
Leucine	70.5 (0.5)	68.6 (0.7)
Tyrosine	9.3[c]	8.5[c]
Phenylalanine	31.8 (1.3)	32.6 (0.2)

[a] Amino acids are expressed as the number of residues per hemoglobin tetramer obtained as average of protein hydrolysis with 6N HCl at 110°C for 24, 48 and 72 hours. Numbers in parentheses are the average deviations.

[b] Value from hydrolysis with 4N methane sulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 115°C for 24 hours.

[c] The 24-, 48-, and 72-hour values for threonine, serine and tyrosine are extrapolated to zero time.

[d] Value from 24 hour 6N HCl hydrolysis after performic acid oxidation. Cysteine determined as cysteic acid. Methionine determined as methionine sulphone.

[e] The 72 hour values are taken for valine and isoleucine.

after acid hydrolysis but can be quantitatively recovered as methionine sulphone after performic acid oxidation (Hirs, 1967). The values for highter lysine content and lower aspartic content might account for the more basic isoelectric point of hemoglobin 1. Due to the presence of non-haem protein, the values for each amino acid of the two hemoglobins may not necessarily be considered as the sum of each residues from individual chains.

Separation of Guinea Pig Hemoglobins into α and β Chains

Figure 5 shows the result of chromatography, at pH 6.7 on CM-cellulose, of globin prepared from total guinea pig hemoglobin. By use of a linear Na^+-ion gradient from 0.005M to 0.03M in 8M urea, total globin was separated into at least three different chains. This preliminary separation gave only a rough indication of the number of different α and β chains that can be expected. A more satisfactory separation was achieved by using longer CM-cellulose columns. After this preliminary separation, each individual globin prepared from hemoglobin 1 and hemoglobin 2 was then separated on 1 x 40 cm CM-cellulose column under the same conditions. The results are shown in Figure 6 and Figure 7 in which β chains were eluted in front of α chains. Since the two chains revealed good separation it was not necessary to re-chromatograph each chain again. The pooled α and β chains were then freed from urea by passing through a Sephadex G-25 column and freed from formic acid by several lyophilizations before storage at -20°C. The peak after the α chain was probably non-haem protein (Garrick et al., 1975; Chua et al., 1974).

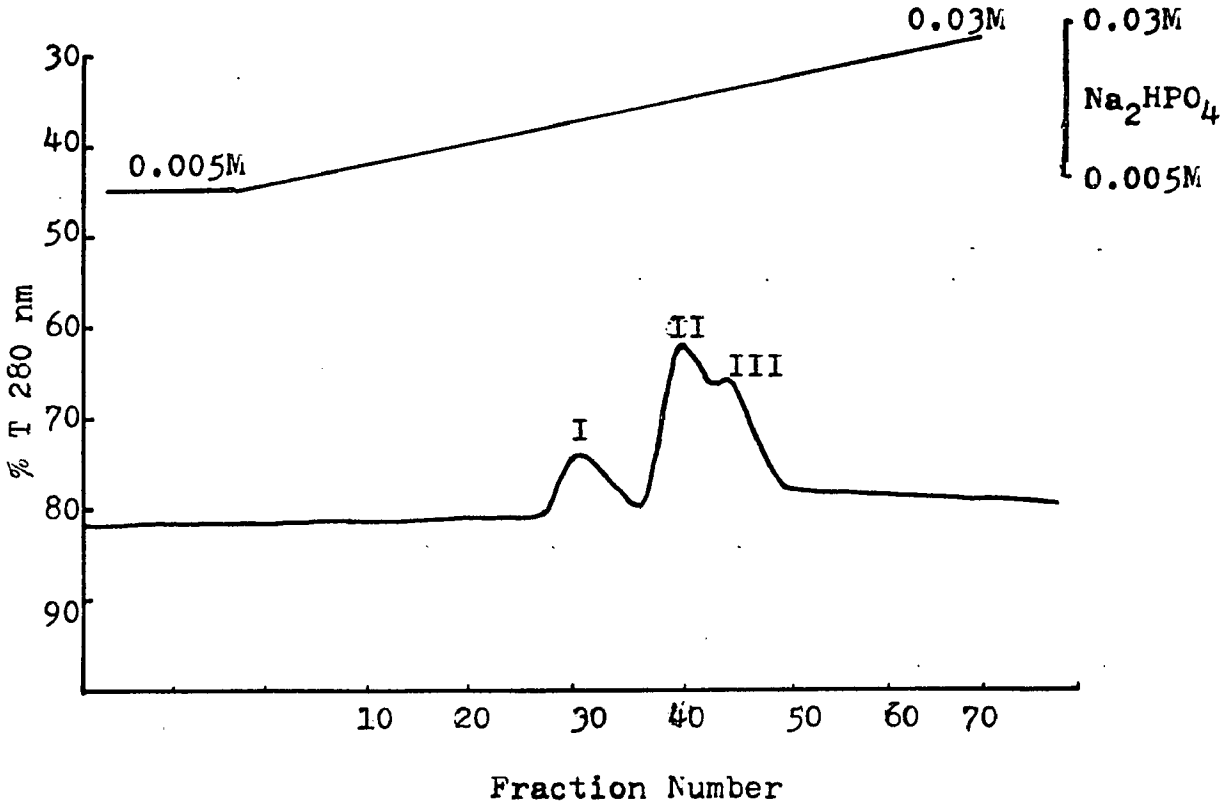


FIGURE 5

SEPARATION OF TOTAL GUINEA PIG GLOBIN INTO α AND β CHAINS

20 mg of total globin was loaded onto a 1 x 10 cm column and 50 ml of starting buffer was passed through it before a Na_2HPO_4 buffer gradient was used. 100 ml of 0.005 M and 100 ml of 0.03 M in 8 M urea with 0.05 M β -mercaptoethanol (pH 6.7).

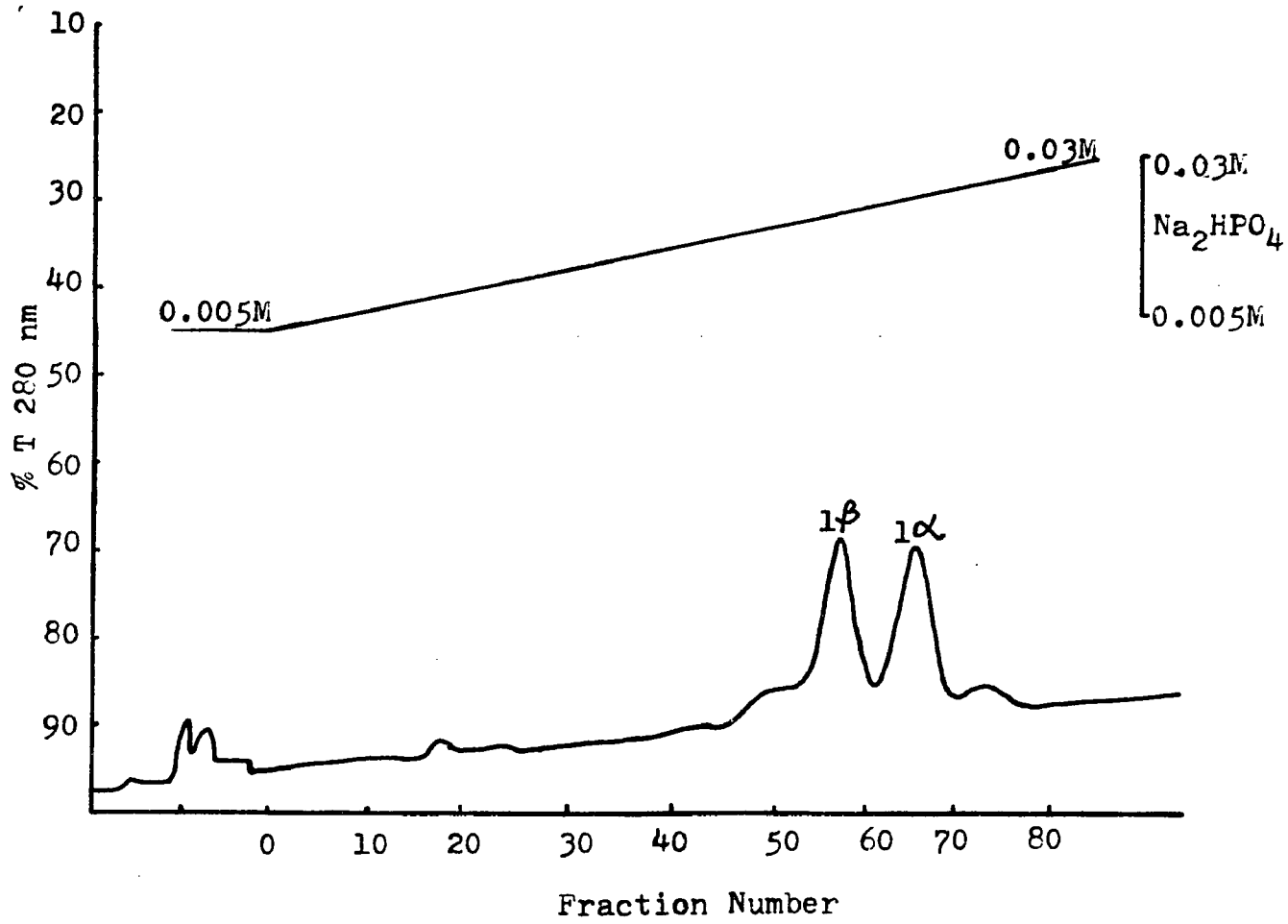


FIGURE 6

SEPARATION OF α-AND β-CHAIN OF GUINEA PIG HEMOGLOBIN #1

40 mg of globin was loaded onto a 1 x 40 cm column and 50 ml of starting buffer was passed through it before a Na_2HPO_4 buffer gradient was used. 150 ml of 0.005 M and 150 ml of 0.03 M in 8 M urea with 0.05 M β-mercaptoethanol (pH 6.7).

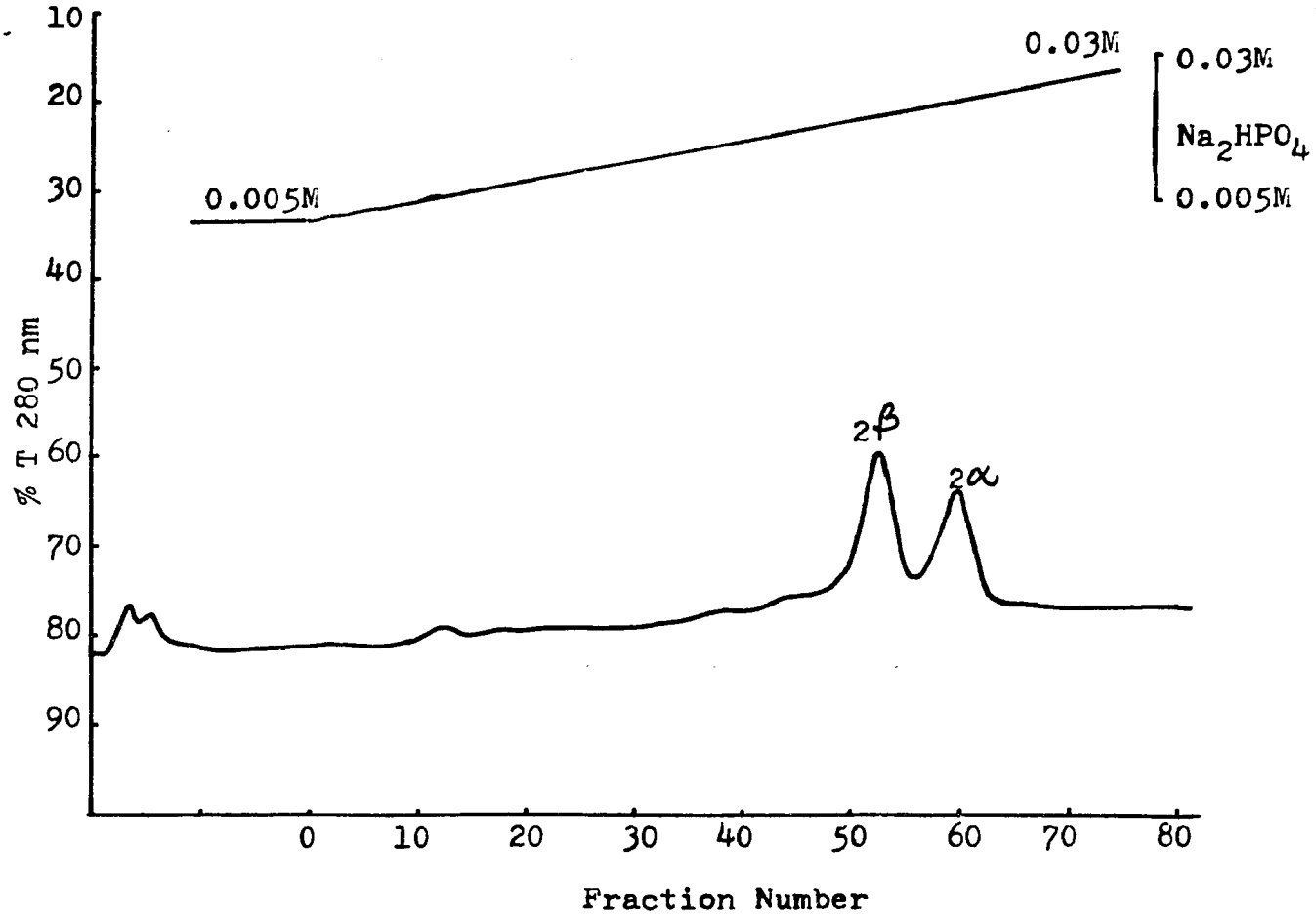


FIGURE 7

SEPARATION OF α-AND β-CHAIN OF GUINEA PIG HEMOGLOBIN #2

40 mg of globin was loaded onto a 1 x 40 cm column and 50 ml of starting buffer was passed through it before a Na_2HPO_4 buffer gradient was used. 150 ml of 0.005 M and 150 ml of 0.03 M in 8 M urea with 0.05 M β-mercaptoethanol (pH 6.7)

Amino Acid Composition of the Chains

The total number of amino acids for each of the two α chains was found to be 141 and the total for two β chains was 146. These results are shown in Table VI and Table VII. From the protein hydrolysate data, differences between α and β chains can be identified.

Amino acid analyses of the two α and two β chains revealed differences. For the α chains there were one amino acid residue differences in the residues of lysine, histidine, threonine, glycine and tyrosine. The most significant differences occuring between the α chains involved the residues aspartic acid (asp and asn) and glutamic acid (glu and gln). 1α contains two less total aspartic acids and three more total glutamic acids than 2α chain (Table VIa). Comparing the differences in the content of basic, neutral and acidic amino acids in the β chains, the differences between two β chains can be seen only in the neutral and acidic amino acids. There were six instances of differences in one amino acid residue, namely, aspartic acid, glycine, alanine, valine, leucine and tyrosine (Table VIIA)

In summary, there are significant differences in overall amino acid composition between the α chains and between the β chains to suggest that two α chains differ from each other as do the two β chains. In order to confirm this, tryptic peptide map comparisons of each of the α and β chains were made and amino acid analysis of tryptic peptides were performed.

TABLE VI

AMINO ACID COMPOSITION OF THE α CHAINS

Amino Acid	1 α		2 α	
	Average[a]		Average[a]	
Tryptophan	0.8[b]	1	1.0[b]	1
Lysine	11.9 (1.1)	12	11.0 (0.2)	11
Histidine	10.7 (0.6)	10	10.6 (0.2)	11
Arginine	2.8 (0.2)	3	2.9 (0.1)	3
Aspartic	11.1 (0.2)	11	13.3 (0.1)	13
Threonine	8.8[c]	9	10.4[c]	10
Serine	10.4[c]	10	9.8[c]	10
Glutamic	10.4 (0.5)	10	7.2 (0.0)	7
Proline	3.6 (1.1)	4	3.7 (0.3)	4
Glycine	9.7 (0.4)	10	9.1 (0.0)	9
Alanine	18.4 (0.5)	18	17.6 (0.3)	18
Cysteine	1.1[d]	1	1.3[d]	1
Valine	11.3[e]	11	11.4[e]	11
Methionine	1.2[d]	1	0.9[d]	1
Isoleucine	4.1[e]	4	4.2[e]	4
Leucine	17.0 (0.7)	17	17.3 (0.2)	17
Tyrosine	2.3[c]	2	2.6[c]	3
Phenylalanine	7.3 (0.3)	7	7.1 (0.2)	7
Total		141		141

[a] The values shown were obtained as average of protein hydrolysis with 6N HCl at 110°C for 24, 48 and 72 hours. Numbers in parentheses are the average deviations.

[b] Value from hydrolysis with 4N methane sulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 115°C for 24 hours.

[c] The 24-, 48-, and 72-hour values for threonine, serine and tyrosine are extrapolated to zero time.

[d] Value from 24 hour 6N HCl hydrolysis after performic acid oxidation. Cysteine determined as cysteic acid. Methionine determined as methionine sulphone.

[e] The 72 hour values are taken for valine and isoleucine.

TABLE VII

AMINO ACID COMPOSITION OF THE β CHAINS

Amino Acid	1 β		2 β	
	Average[a]		Average[a]	
Tryptophan	1.4[b]	1	1.2[b]	1
Lysine	11.1 (0.3)	11	10.7 (0.2)	11
Histidine	10.2 (0.3)	10	9.9 (0.2)	10
Arginine	3.2 (0.1)	3	3.0 (0.1)	3
Aspartic	12.0 (0.9)	12	11.3 (0.1)	11
Threonine	7.2[c]	7	7.4[c]	7
Serine	10.3[c]	10	10.2[c]	10
Glutamic	12.0 (0.3)	12	12.1 (0.3)	12
Proline	3.7 (0.7)	4	4.1 (0.1)	4
Glycine	10.3 (0.5)	10	10.5 (0.1)	11
Alanine	19.1 (0.4)	19	19.9 (0.3)	20
Cysteine	0.8[d]	1	1.4[d]	1
Valine	11.1[e]	11	10.4[e]	10
Methionine	1.7[d]	2	1.8[d]	2
Isoleucine	4.9[e]	5	4.8[e]	5
Leucine	17.9 (0.4)	18	18.9 (0.2)	19
Tyrosine	1.9[c]	2	1.4[c]	1
Phenylalanine	7.9 (0.5)	8	7.7 (0.2)	8
Total		146		146

[a] The values shown were obtained as average of protein hydrolysis with 6N HCl at 110°C for 24, 48 and 72 hours. Numbers in parentheses are the average deviations.

[b] Value from hydrolysis with 4N methane sulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 115°C for 24 hours.

[c] The 24-, 48-, and 72-hour values for threonine, serine and tyrosine are extrapolated to zero time.

[d] Value from 24 hour 6N HCl hydrolysis after performic acid oxidation. Cysteine determined as cysteic acid. Methionine determined as methionine sulphone.

[e] The 72 hour values are taken for valine and isoleucine.

TABLE VIA

DIFFERENCES IN THE AMINO ACID COMPOSITIONS OF THE α CHAINS
(from Table VI)

Amino Acid	1α	2α	Difference
Lysine	12	11	1α has one more lysine than 2α.
Histidine	10	11	1α has one less histidine than 2α.
Aspartic	11	13	1α has two less total aspartic than 2α.
Threonine	9	10	1α has one less threonine than 2α.
Glutamic	10	7	1α has three more total glutamic than 2α.
Glycine	10	9	1α has one more glycine then 2α.
Tyrosine	2	3	1α has one less tyrosine than 2α.

TABLE VIIA

DIFFERENCES IN THE AMINO ACID COMPOSITIONS OF THE β CHAINS
(from Table VII)

Amino Acid	1β	2β	Difference
Aspartic	12	11	1β has one more total aspartic than 2β.
Glycine	10	11	1β has one less glycine than 2β.
Alanine	19	20	1β has one less alanine than 2β.
Valine	11	10	1β has one more valine than 2β.
Leucine	18	19	1β has one less leucine than 2β.
Tyrosine	2	1	1β has one more tyrosine than 2β.

Two-Dimensional Separation of Tryptic Peptides

The patterns of peptides obtained by two-dimensional paper electrophoresis and chromatography are shown in Figure 8 and Figure 9 where the peptides are numbered arbitrarily. It was found that descending chromatography followed by high voltage paper electrophoresis gave the best separation of the peptides in each "fingerprint" map. The number of peptides expected after trypsin digestion is one more than the total number of lysine and arginine in the protein. But guinea pig α chains, like other mammalian α chains, contain arginine as a C-terminal residue and thus account for one less peptide than expected. Therefore, 15 peptides was predicted in the 1α chain and 14 peptides was predicted in the 2α chain. For the two β chains the 15 peptide spots obtained were predicted. Comparison of the pattern of 1α and 2α peptides and 1β and 2β peptides shows that there is great similarity in both electrophoretic mobility and R_f values of many of the peptides. This suggested that there must be a similarity in amino acid sequence in these peptides.

The peptide spots were first located by spraying a map with 0.5% ninhydrin in 95% alcohol. The same maps were then followed by specific stains for histidine and tyrosine-Pauly reagent. A deep cherry red color revealed spots containing histidine or tyrosine. In the respective α peptide maps tryptic peptides which gave a positive Pauly reaction were $1\alpha 2$, $1\alpha 4$, $1\alpha 6$, $1\alpha 8$, $1\alpha 10$, $1\alpha 13$, $1\alpha 14$; and $2\alpha 2$, $2\alpha 4$, $2\alpha 6$, $2\alpha 9$, $2\alpha 10$, $2\alpha 12$, $2\alpha 13$. In each β chain map, six tryptic peptides gave a positive

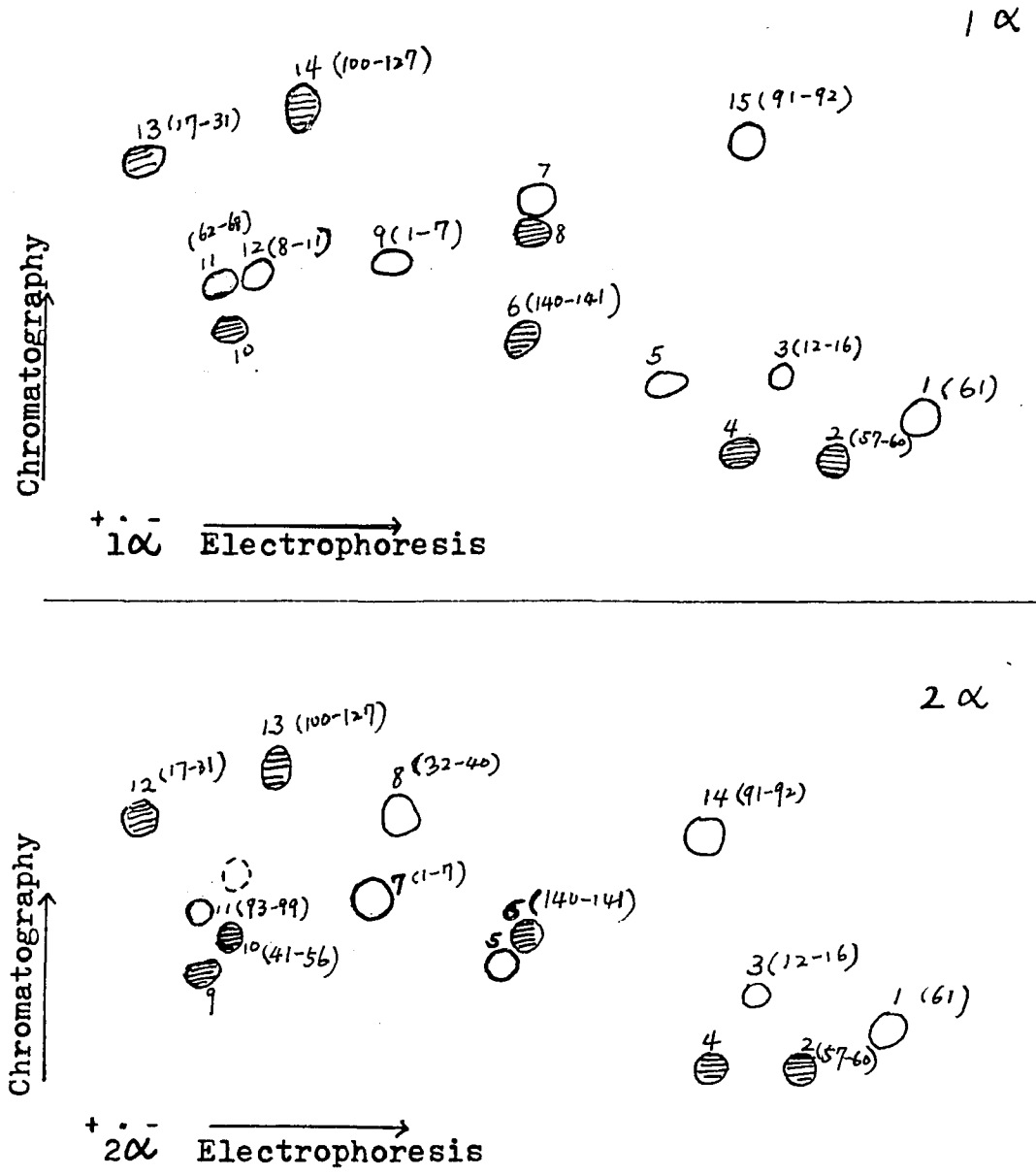
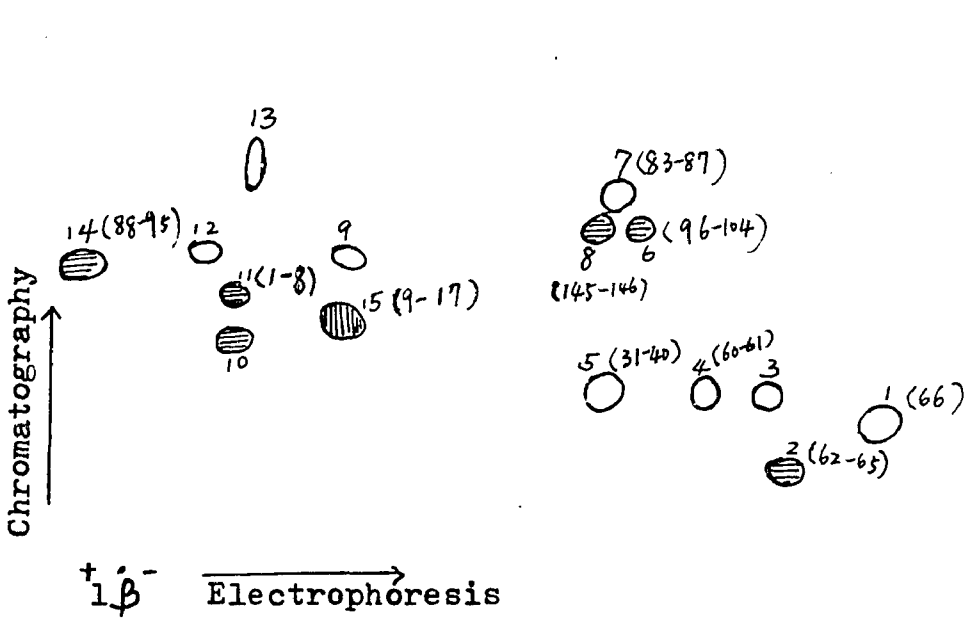


FIGURE 8

TRYPTIC PEPTIDE MAPS OF THE α CHAINS

Descending chromatography was carried out in n-butanol-acetic acid-water(4:1:5 by volume) followed by electrophoresis in the second dimension in pyridine-acetic acid-water (10:0.4:90 by volume; pH 6.4). ⊜ = Positive Pauly reaction for histidine and/or tyrosine. Numbers in () indicate that portion of the α chain sequence the peptide represents.



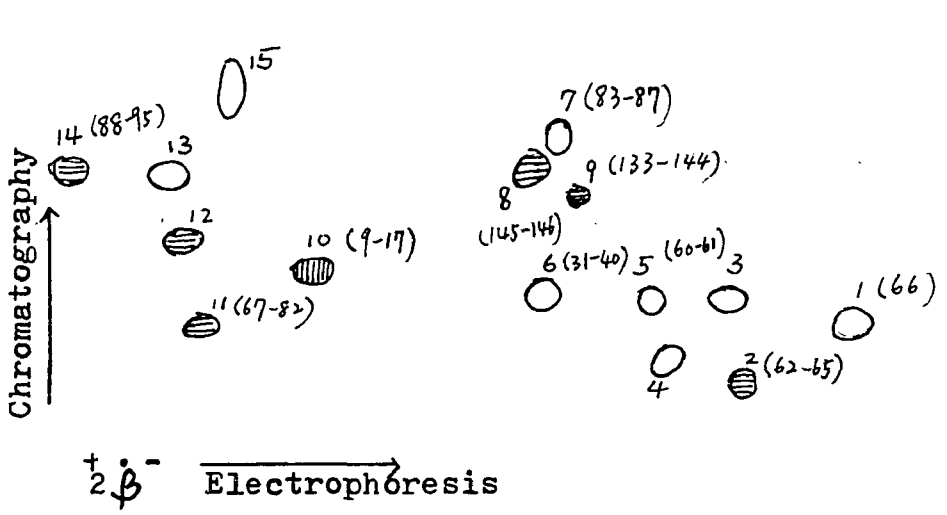


FIGURE 9

TRYPTIC PEPTIDE MAPS OF THE β CHAINS

Descending chromatography was carried out in n-butanol-acetic acid-water (4:1:5 by volume) followed by electrophoresis in the second dimension in pyridine-acetic acid-water (10:0.4:90 by volume; pH 6.4). ◎ = Positive Pauly reaction for histidine and/or tyrosine. ◍ = Positive Ehrlich reaction for tryptophan. Numbers in () indicate that portion of the β chain sequence the peptide represents.

Pauly reaction. They were 1β2, 1β6, 1β8, 1β10, 1β11, 1β14; 2β2, 2β8, 2β9, 2β11, 2β12, 2β14. The contradiction of the number of spots which gave positive Pauly reaction and the total number of histidine and tyrosine in each chain may be explained as follows:

1) Some of these peptides contained more than one histidine, tyrosine or histidine and tyrosine.
2) Peptides which did not give a positive Pauly reaction nevertheless contained histidine or tyrosine.

A second peptide map was used to detect tryptophan containing spots by use of the Ehrlich reagent. A positive Ehrlich reaction gave a purple color. In Figure 9 it can be seen that one peptide in each of β maps gave a positive Ehrlich reaction. They are 1β15 and 2β10. No positive Ehrlich spot was shown in any of the α maps though Table VI indicates there is one tryptophan per α chain. This might be due to insensitivity of Ehrlich reagent applied to a limited quantity of tryptophan in the peptide spot.

A preliminary comparison of the tryptic digest maps shows that many of the peptide spots are common in their location in each set of chains. There are nine peptide spots common to all α chains, ten peptide spots common to all β chains. Eleven peptides- six from 1α and five from 2α- which do not have matching counterparts in the other map and are therefore unique to that particular α chain. In the same way ten peptides-five from 1β and five from 2β-are unique to

β chains. A summary of these are shown in Table VIIIa and Table VIIIb. Based on comparison of individual peptide maps the conclusion can be drawn that there are two different α chains and two different β chains in the guinea pig hemoglobins. Supporting evidence from amino acid composition studies of peptides will be used to confirm this conclusion.

Amino Acid Composition of Each α and β Chain Peptide

Trypsin splits peptide bonds at the carboxyl side of arginine and lysine. For this reason, when determining the amino acid composition of the tryptic peptides, the molar proportion ratio were always based on the assumption that the lysine (or arginine) present represented one lysine (or arginine) residue per mole. These results are shown in Tables IX, X, XI and XII. An amino acid was judged to be a part of a peptide if it had a molar ratio value of 0.45 or higher. Values of 0.40 were allowed for the amino acids valine, isoleucine and tyrosine due to poorer recovery after hydrolysis.

Sequence Homology

Spots eluted from the peptide maps were hydrolyzed for the determination of their amino acid compositions. The residues were then fitted into a sequence by homology with human, dog, mouse and rabbit globins (Dayhoff, 1972). If the amino acid composition of the peptide was similarly matched to those mammalian globin by homology it was assigned a number by analogy. Many of the peptides could be easily sequenced by homology. However, a few peptides contained amino acids quite unlike the

TABLE VIIIa

TRYPTIC PEPTIDE COMPARISON OF α CHAINS BY MAP POSITIONING
(from Figure 8)

Tryptic Peptides Common to 1α and 2α

$1\alpha 1$	$2\alpha 1$
$1\alpha 2$	$2\alpha 2$
$1\alpha 3$	$2\alpha 3$
$1\alpha 4$	$2\alpha 4$
$1\alpha 6$	$2\alpha 6$
$1\alpha 9$	$2\alpha 7$
$1\alpha 14$	$2\alpha 13$
$1\alpha 15$	$2\alpha 14$
$1\alpha 13$	$2\alpha 12$

Tryptic Peptides Unique to 1α and 2α

$1\alpha 5$, $1\alpha 7$, $1\alpha 8$, $1\alpha 10$, $1\alpha 11$, $1\alpha 12$

$2\alpha 5$, $2\alpha 8$, $2\alpha 9$, $2\alpha 10$, $2\alpha 11$

TABLE VIIIb

TRYPTIC PEPTIDE COMPARISON OF β CHAINS BY MAP POSITIONING
(from Figure 9)

Tryptic Peptides Common to 1β and 2β

$1\beta 1$	$2\beta 1$
$1\beta 2$	$2\beta 2$
$1\beta 3$	$2\beta 3$
$1\beta 4$	$2\beta 5$
$1\beta 5$	$2\beta 6$
$1\beta 7$	$2\beta 7$
$1\beta 8$	$2\beta 8$
$1\beta 13$	$2\beta 15$
$1\beta 14$	$2\beta 14$
$1\beta 15$	$2\beta 10$

Tryptic Peptides Unique to 1β and 2β

$1\beta 6$, $1\beta 9$, $1\beta 10$, $1\beta 11$, $1\beta 12$; $2\beta 4$, $2\beta 9$, $2\beta 11$, $2\beta 12$, $2\beta 13$

TABLE IX
AMINO ACID COMPOSITION OF THE 1α CHAIN TRYPTIC PEPTIDES[a]

Amino Acid	1α1	1α2	1α3	1α4	1α5	1α6	1α7	1α8	1α9	1α10	1α11	1α12	1α13	1α14	1α15
Lysine	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00		1.00	
Histidine		1.15		0.75				2.15		0.61			0.70	++++	
Arginine				0.10		1.00							1.00		1.00
Aspartic			0.18	0.03	0.17					1.15		1.21	0.52	2.29	
Threonine						0.12	1.00		0.90		0.95	1.37		1.47	
Serine			0.20	0.85						0.66	1.28	0.47	0.70	0.80	
Glutamic			0.16		1.25	0.21	0.07		0.90	0.70	1.03	0.17	0.65	1.06	
Proline										0.20			0.30	0.91	
Glycine	0.02	0.91	0.85	1.17	0.28	0.07	0.91		0.25	0.45	0.69	0.31	0.74	1.72	
Alanine	0.02	0.80	1.56	1.09		0.09	1.20	1.12	1.95	0.57	0.84	0.36	0.66	2.75	
Cysteine															
Valine									0.76	0.40	1.35	0.47	0.47	0.96	
Methionine														0.75	
Isoleucine										0.15			0.43		
Leucine			0.78				0.26		0.88	0.90	0.89	0.56	0.56	0.20	0.93
Tyrosine						0.72							0.14		
Phenylalanine	0.01						0.91			0.12			0.25	0.89	

[a]Hydrolysis with 6N HCL at 110°C for 24 hours.
The values are mole ratios determined by amino acid analysis.

++++ indicates amino acid is positively stained by Pauly reagent but is unresolved.

TABLE X
AMINO ACID COMPOSITION OF THE 2α CHAIN TRYPTIC PEPTIDES[a]

Amino Acid	2α1	2α2	2α3	2α4	2α5	2α6	2α7	2α8	2α9	2α10	2α11	2α12	2α13	2α14
Lysine	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00		1.00	0.25
Histidine		0.98		0.45					++++	1.87		1.21	1.21	
Arginine						1.00	0.13					1.00		1.00
Aspartic									0.92	1.79	1.83	1.18	2.33	0.19
Threonine			0.25		1.59		0.88	2.04	0.18	0.60	0.20	1.10	1.57	0.20
Serine								0.78	0.45	2.26		0.80	0.50	
Glutamic			0.08				0.52		0.38		0.26	1.37	0.56	0.11
Proline								0.50		1.19	0.46	0.37	0.97	
Glycine	0.08	1.07	0.92	0.47	0.96				0.96	2.31		2.45	1.00	0.26
Alanine		0.84	1.78	0.83	1.58		1.94		0.43			3.61	3.39	0.32
Cysteine														
Valine						0.11	0.67		0.19	1.28	0.70		1.39	
Methionine								0.55						
Isoleucine									0.10	1.29	0.21	0.84	0.18	
Leucine			0.82	0.37			0.91		0.59		0.92	1.72	1.43	0.90
Tyrosine						0.54				1.03	0.12	0.61	0.47	
Phenylalanine					0.76	0.43		2.05		1.13	0.54	0.36	0.35	

[a]Hydrolysis with 6N HCL at 110°C for 24 hours.
++++ indicates amino acid is positively stained by Pauly reagent but is unresolved.
The values are mole ratios determined by amino acid analysis.

TABLE XI
AMINO ACID COMPOSITION OF THE 1β CHAIN TRYPTIC PEPTIDES[a]

Amino Acid	1β1	1β2	1β3	1β4	1β5	1β6	1β7	1β8	1β9	1β10	1β11	1β12	1β13	1β14	1β15
Lysine	1.00	1.00	1.00	1.00			1.00		1.00		1.00		1.00	1.00	1.00
Histidine		0.89				0.80		1.03		0.73	0.66			0.81	0.29
Arginine					1.00	1.00						1.00			
Aspartic			0.23	0.13		1.00	0.20			1.12	1.30	2.67	0.07	1.30	0.10
Threonine	0.07				0.79	0.68	0.43		1.06	0.21	0.46	0.92		0.25	0.97
Serine		0.21		0.13						0.85	0.96	1.26	0.39	0.39	
Glutamic	0.03			1.03	1.03		0.21		1.12	0.99	1.06	1.55	1.01	1.34	1.11
Proline						0.56					0.19				
Glycine		1.17	0.72			0.10	0.60			0.23	0.50	1.56	0.26	0.39	
Alanine		1.16	1.89			0.93	1.20		2.12	0.36	0.80	2.48	0.26		3.05
Cysteine															
Valine						1.24	0.24			0.21	0.46	2.19	0.25	0.39	
Methionine														0.08	
Isoleucine										0.09	0.24	0.33	0.04	0.24	
Leucine			0.77			0.77	0.18		1.06	1.25	0.97	1.22	0.46	2.27	1.11
Tyrosine							0.49	0.59							
Phenylalanine							0.54				0.20	0.93	1.77	0.28	

[a]Hydrolysis with 6N HCL at 110°C for 24 hours.
The values are mole ratios determined by amino acid analysis.

TABLE XII
AMINO ACID COMPOSITION OF THE 2β CHAIN TRYPTIC PEPTIDES[a]

Amino Acid	2β1	2β2	2β3	2β4	2β5	2β6	2β7	2β8	2β9	2β10	2β11	2β12	2β13	2β14	2β15
Lysine	1.00	1.00	1.00	1.00	1.00		1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00
Histidine		1.01					0.10	1.22	0.94	0.22	0.72	1.00		0.62	
Arginine						1.00	0.10						1.10		
Aspartic							0.24		0.69	0.15	3.12	2.27	3.14	0.86	
Threonine				0.63		0.45	0.95	0.03	1.36	1.08	0.25	0.93	1.34		
Serine							0.24			0.08	1.92	1.48	1.70	0.76	
Glutamic						0.92	0.33	0.03		1.20	2.82	1.28	1.46	0.77	1.56
Proline							0.14						0.91		
Glycine		0.76	1.02				0.95	0.05	1.26	0.25	0.86	0.83	1.83	0.12	0.21
Alanine		0.89	2.12	0.91			1.29	0.05	4.28	2.06	0.58	2.15	3.74	0.17	2.51
Cysteine															
Valine					0.92				2.75				2.69	0.15	
Methionine															
Isoleucine				1.02			0.10					0.61	0.71		
Leucine			0.62				0.29		1.39	1.23	3.87	2.36	2.74	1.50	
Tyrosine								1.00							
Phenylalanine							0.90						1.36		

[a]Hydrolysis with 6N HCL at 110°C for 24 hours.
The values are mole ratios determined by amino acid analysis.

known sequence of those species, and therefore cannot be sequenced. Some peptides which were in the insoluble "core" could not be separated by peptide "mapping" and this would account for one of the reasons for missing fragments from the chains during sequencing.

Amino acid substitutions in globin chains have been reported quite often in the different strain of animals or in the different individual of the same strain e.g., Glu →Val (Ingram, 1958), Val →Leu, Leu →Phe, Ser →Thr, Val →Thr, Leu →Val, Ser →Leu (Ehrenstein, 1966) and Ala →Asp (Romain et al., 1975). In addition some positions in the globin chains have also been found to contain additional amino acids (Ehrenstein, 1966). These phenomena were useful for sequencing. Glutamine and asparagine residues are written as "Glx" and "Asx" respectively.

Sequence Homology of the α Chains -Residue 61

Tryptic peptides were assumed to be common and to have the same amino acid composition if they matched each other by position in the peptide maps. "Peptides" 1α1 and 2α1 appeared in the same positions on their respective maps (Figure 8, Table VIIIa). They also contained the same and only one amino acid lysine. By sequence homology these peptides represent residue 61 in α chain.

Species	61
Human α	Lys
Dog α	Lys
Mouse α	Lys
Rabbit α	Lys
1 α 1	Lys
2 α 1	Lys

Sequence Homology of the α Chains - Residues 57-60

Common peptides 1 α 2 and 2 α 2 (Figure 8, Table VIIIa) all contain the amino acids lysine, histidine, glycine and alanine. By homology the best fitted positions are 57-60.

Species	57 58 59 60
Human α	Gly-His-Gly-Lys
Dog α	Ala-His-Gly-Lys
Mouse α	Gly-His-Gly-Lys
Rabbit α	Ala-His-Gly-Lys
1 α 2	Ala His Gly Lys
2 α 2	Ala His Gly Lys

Sequence Homology of the α Chains - Residues 12-16

In Table VIIIa, common peptides 1 α 3 and 2 α 3 were found to contain the same five amino acids - lysine, glycine, two alanines and leucine.

Species	12 13 14 15 16
Human α	Ala-Ala-Trp-Gly-Lys
Dog α	Ser-Thr-Trp-Asp-Lys
Mouse α	Ala-Ala-Trp-Gly-Lys
Rabbit α	Thr-Ala-Trp-Gly-Lys
1 α 3	Ala Ala Leu Gly Lys
2 α 3	Ala Ala Leu Gly Lys

From homology study, these peptides appear to match positions 12-16 in α chain except position 14. Position 14

in the sequence is indicated to be tryptophan but these two peptide spots did not show positive Ehrlich reaction nor could tryptophan be detected due to acid hydrolysis. In addition, 1α3 and 2α3 contain leucine. If this leucine was not from contamination it is possible that leucine and tryptophan are exchanged in this case.

Sequence Homology of the α Chains - Residues 1-7

Peptides 1α9 and 2α7 were located in the same position on their respective maps. Both amino acid composition and sequence homology studies put these two peptides into the first segment of the α chain - residues 1-7.

Species	1	2	3	4	5	6	7
Human α	Val	Leu	Ser	Pro	Ala	Asp	Lys
Dog α	Val	Leu	Ser	Pro	Ala	Asp	Lys
Mouse α	Val	Leu	Ser	Gly	Glu	Asp	Lys
Rabbit α	Val	Leu	Ser	Pro	Ala	Asp	Lys
1 α9	Val	Leu	Thr	Ala	Ala	Glx	Lys
2 α7	Val	Leu	Thr	Ala	Ala	Glx	Lys

Sequence Homology of the α Chains - Residues 140-141

Peptides 1α6 and 2α6 were listed as common peptides by map location (Fifure 8, Table VIIIa). After amino acid analysis these two peptides appeared to contain two amino acids - tyrosine and arginine. In additions, both peptides were shown to give a positive Pauly reaction. By homology 1 α6 and 2 α6 represent residues 140-141, the last two amino acids of the chains.

Species	140	141
Human α	Tyr-	Arg
Dog α	Tyr-	Arg
Mouse α	Tyr-	Arg
Rabbit α	Tyr-	Arg
1 α 6	Tyr	Arg
2 α 6	Tyr	Arg

Sequence Homology of the α Chains - Residues 91-92

Peptides 1 α15 and 2 α14 were another set of common peptide spots by map positioning (Figure 8, Table VIIIa). Residues 91-92 on the α chain sequence were found to be located in these two peptides since two amino acids - leucine and arginine were shown after analysis.

Species	91	92
Human α	Leu-	Arg
Dog α	Leu-	Arg
Mouse α	Leu-	Arg
Rabbit α	Leu-	Arg
1 α15	Leu	Arg
2 α14	Leu	Arg

Sequence Homology of the α Chains - Residues 17-31

Tryptic peptides 1 α13 and 2 α12 have the same map position and positive Pauly reaction (Figure 8), yet their total amino acid compositions appear to be slightly different since 1 α13 was short by one tyrosine, two alanines and two glycines. 2 α12 is well matched in residues 17-31 on the α chain except for leaving a Glu residue blank. This Glu residue might not have enough time to release due to the influence of the bulky leucine residue next to it. The

shortage of amino acids in 1α13 does not necessarily mean they are not present in the peptide. It could be that 1α13 did not release its amino acids as well as 2α12.

Species	17	18	19	20	21	22	23	24	25	26	27	28
Human α	Val-	Gly-	Ala-	His-	Ala-	Gly-	Glu-	Tyr-	Gly-	Ala-	Glu-	Ala-
Dog α	Ile-	Gly-	Gly-	His-	Ala-	Gly-	Asp-	Tyr-	Gly-	Gly-	Glu-	Ala-
Mouse α	Ile-	Gly-	Gly-	His-	Gly-	Ala-	Glu-	Tyr-	Gly-	Ala-	Glu-	Ala-
Rabbit α	Ile-	Gly-	Ser-	His-	Gly-	Gly-	Glu-	Tyr-	Gly-	Ala-	Glu-	Ala-
1α13	Val	Gly	Ser	His			Asx				Glx	Ala
2α12	Ile	Gly	Ser	His	Ala	Gly	Asx	Tyr	Gly	Ala	Glx	Ala

Species	29	30	31
Human α	Leu-	Glu-	Arg
Dog α	Leu-	Glu-	Arg
Mouse α	Leu-	Glu-	Arg
Rabbit α	Val-	Glu-	Arg
1α13	Leu		Arg
2α12	Leu		Arg

Sequence Homology of the α Chains - Residues 100-127

Peptides 1α14 and 2α13 located at the same position on their map (Figure 8). The amino acid composition of these peptides indicates that they contain almost the same amino acids (Table IX and Table IX) and that they represent the same sequence segment in α chain - residues 100-127.

Species	100	101	102	103	104	105	106	107	108	109	110
Human α	Leu-	Leu-	Ser-	His-	Cys-	Leu-	Leu-	Val-	Thr-	Leu-	Ala-
Dog α	Leu-	Leu-	Ser-	His-	Cys-	Leu-	Leu-	Val-	Thr-	Leu-	Ala-
Mouse α	Leu-	Leu-	Ser-	His-	Cys-	Leu-	Leu-	Val-	Thr-	Leu-	Ala-
Rabbit α	Leu-	Leu-	Ser-	His-	Cys-	Leu-	Leu-	Val-	Thr-	Leu-	Ala-
1α14								Val	Thr		Ala
2α13								Val	Thr		Ala

Species	111	112	113	114	115	116	117	118	119	120	121
Human α	Ala-	His-	Leu-	Pro-	Ala-	Glu-	Phe-	Thr-	Pro-	Ala-	Val-
Dog α	Cys-	His-	His-	Pro-	Thr-	Gly-	Phe-	Thr-	Pro-	Ala-	Val-
Mouse α	Ser-	His-	His-	Pro-	Ala-	Asp-	Phe-	Thr-	Pro-	Ala-	Val-
Rabbit α	Asn-	His-	Val-	Pro-	Ser-	Glu-	Phe-	Thr-	Pro-	Ala-	Val-
1 α14	Asx					Gly	Glx	Phe	Thr	Pro	Ala	Met
2 α13	Asx					Gly	Glx		Thr	Pro	Ala	Val

Species	122	123	124	125	126	127
Human α	His-	Ala-	Ser-	Leu-	Asp-	Lys
Dog α	His-	Ala-	Ser-	Leu-	Asp-	Lys
Mouse α	His-	Ala-	Ser-	Leu-	Asp-	Lys
Rabbit α	His-	Ala-	Ser-	Leu-	Asp-	Lys
1 α14	His	Ala	Ser	Leu	Asx	Lys
2 α13	His	Ala	Ser	Leu	Asx	Lys

These two peptides represent one of the longest segments found in the α chain - 28 amino acids. Because these peptides were so long, not all of the amino acids in these peptides were recovered, especially those residues which contain bulky side chains vicinity. In peptide 1 α14, position 121 was methionine and this situation has been reported in rat αchains (Ranney et al, 1975; Chua et al., 1975). Those fragments which did not show up in amino acid analysis might have remained in the insoluble "core".

Amino Acid Composition Comparison - Peptides 1 α4 and 2 α4

The last set of α tryptic peptides to be discussed are the 1 α4 and 2 α4 peptides. They have the same map locations (Figure 8) and contain the same amino acids - lysine, histidine, serine, glycine and alanine. Two possibilities can be drawn for these two peptides:

1) They stand for residues 57-60, the same as peptides 1 α 2 and 2 α2 with serine as an extra amino acid.
2) These two peptides could not be placed by homology into the α chain sequence and might come from some fragment of a larger peptide.

Sequence Homology of the αChain - for Some Unique Peptides

Some unique peptides e.g., 1 α11, 1α12, 2 α8, 2α10 and 2 α11 which did not have counterparts in the other map can be sequenced for the α chain by homology. They are discussed in the following sections.

Sequence Homology of the 1α Chain - Peptides 1 α11 and 1 α12

Residues 62-68 on the αchain sequence were located in peptide 1 α11. The amino acid composition of 1 α11 indicates that it contains seven amino acids - lysine, threonine, serine, glutamic, alanine, valine and leucine. It appears to match positions 62-68 on the αchain by homology with other known α chain sequences. Peptide 1 α11 is a unique segment in the 1α chain, not only because the same sequence cannot be found in any 2α peptide but the lysine residue is at position 68, the same as in the rabbit α chain. Other species cited here have their lysine residue of this segment at position 90.

Species	62	63	64	65	66	67	68
Human α	Val-	Ala-	Asp-	Ala-	Leu-	Thr-	Asn
Dog α	Val-	Ala-	Asp-	Ala-	Leu-	Thr-	Thr
Mouse α	Val-	Ala-	Asp-	Ala-	Leu-	Ala-	Asn
Rabbit α	Val-	Ser-	Gln-	Ala-	Leu-	Thr-	Lys
1 α11	Val	Ser	Glx	Ala	Leu	Thr	Lys

Another unique peptide spot in the 1α map is 1α12. It contains six amino acids and seems to fit residues 8-11 in the α chain but with two extra amino acids-serine and leucine. Since serine is often exchanged with threonine, the same as for leucine with valine, serine and leucine might be counted as one of the double amino acids in position 8 and 10 on the α chain respectively, i.e., a mixture of two unseparated peptides.

Species	8	9	10	11
Human α	Thr-	Asn-	Val-	Lys
Dog α	Thr-	Asn-	Ile-	Lys
Mouse α	Ser-	Asn-	Ile-	Lys
Rabbit α	Thr-	Asn-	Ile-	Lys
1 α12	Thr Ser	Asx	Val Leu	Lys

Sequence Homology of the 2α Chain - Peptides 2α8, 2α10 and 2α11

Tryptic peptides 2α8, 2α10 and 2α11 are peptides which can be sequenced on the α chain by homology among the unique 2α peptide spots (Table VIIIa). By homology, 2α8 represents residues 32-40, 2α10 represents residues 41-56 and 2α11 represents residues 93-99.

Species	32	33	34	35	36	37	38	39	40
Human α	Met-	Phe-	Leu-	Ser-	Phe-	Pro-	Thr-	Thr-	Lys
Dog α	Thr-	Phe-	Gln-	Ser-	Phe-	Pro-	Thr-	Thr-	Lys
Mouse α	Met-	Phe-	Ala-	Ser-	Phe-	Pro-	Thr-	Thr-	Lys
Rabbit α	Met-	Phe-	Leu-	Gly-	Phe-	Pro-	Thr-	Thr-	Lys
2 α8	Met	Phe		Ser	Phe	Pro	Thr	Thr	Lys

Species	41	42	43	44	45	46	47	48	49	50	51
Human α	Thr-	Tyr-	Phe-	Pro-	His-	Phe-	Asp-	Leu-	Ser-	His-	Gly-
Dog α	Thr-	Tyr-	Phe-	Pro-	His-	Phe-	Asp-	Leu-	Ser-	Pro-	Gly-
Mouse α	Thr-	Tyr-	Phe-	Pro-	His-	Phe-	Asp-	Val-	Ser(	His	Gly
Rabbit α	Thr-	Tyr-	Phe-	Pro-	His-	Phe-	Asp-	Phe-	Thr-	His-	Gly-
2 α10	Thr	Tyr	Phe	Pro	His	Phe	Asx	Val	Ser	His	Gly

Species	52	53	54	55	56
Human α	Ser-	Ala-	Gln-	Val-	Lys
Dog α	Ser-	Ala-	Gln-	Val-	Lys
Mouse α	Ser	Ala	Gln	Val)	Lys
Rabbit α	Ser-	Gly-	Gln-	Ile-	Lys
2 α10	Ser	Gly	Asx	Ile	Lys

Species	93	94	95	96	97	98	99
Human α	Val-	Asp-	Pro-	Val-	Asn-	Phe-	Lys
Dog α	Val-	Asp-	Pro-	Val-	Asn-	Phe-	Lys
Mouse α	Val-	Asp-	Pro-	Val-	Asn-	Phe-	Lys
Rabbit α	Val-	Asp-	Pro-	Val-	Asn-	Phe-	Lys
2 α11	Val	Asx	Pro	Leu	Asx	Phe	Lys

α Chain Summary

All the results which arise from sequence homology of α chains can be summarized in Figure 10 which illustrates the partial sequences of the α chains of guinea pig hemoglobins 1 and 2. The positions in the sequence occupied by the tryptic peptides were assigned by homology with hemoglobins from other mammalian species. The differences in the α chains were as follows:

1) Peptides 1 α12 and 1 α11, representing residues 8-11 and 62-68 respectively on the 1 α chain, have no peptide counterparts located on the 2α map. Amino Acid analysis of unique peptides on the 2 α

map also failed to show similiar amino acid compositions.

2) Peptides 2α8, 2α10 and 2α11 can be identified as residues 32-40, 41-56 and 93-99 respectively on the 2α chain. No peptide counterpart can be located on the 1α map nor can the same amino acid sequence be found in the unique peptides of the 1α map.

3) The 1α chain contains valine at position 17, whereas the corresponding peptide from the 2α chain has isoleucine at the same position.

4) A methionine residue is present at position 121 in the 1α chain, but valine is present in the 2α chain.

From this data the conclusion can be drawn that in the guinea pig the 1α and 2α globin chains differ from each other in their amino acid compositions and in their amino acid sequences to a significant extent.

Sequence Homology of the β Chains - Residue 66

An investigation of common "peptides" 1β1 and 2β1 (Figure 9, Table VIIIb) revealed that they contained only one amino acid - lysine. By homology these two peptides represent residue 66 on the β chain sequence.

Species	66
Human β	Lys
Dog β	Lys
Mouse β	Lys
Rabbit β	Lys
1 β 1	Lys
2 β 1	Lys

Sequence Homology of the β Chains - Residues 62-65

Peptides 1 β 2 and 2 β 2 appear to be common peptides by map positioning and positive Pauly reaction. After amino acid analysis the two peptides contained four amino acids - lysine, histidine, glycine and alanine. This corresponds to positions 62-65 on the β chain sequence by homology.

Species	62 63 64 65
Human β	Ala-His-Gly-Lys
Dog β	Ala-His-Gly-Lys
Mouse β	Ala-His-Gly-Lys
Rabbit β	Ala-His-Gly-Lys
1 β 2	Ala His Gly Lys
2 β 2	Ala His Gly Lys

Sequence Homology of the β Chains - Residues 60-61

Tryptic peptides 1 β 4 and 2 β 5 were thought to be the same peptides by map location (Figure 9). After amino acid analysis 1 β 4 contained glutamic acid and lysine whereas 2 β 5 contained valine and lysine. They represent positions 60-61 in the β chain sequence by homology. Since these two peptide spots have the same mobility on the map and position 60 is almost always occupied by a valine which is a nonpolar amino acid 1β 4 is impossible to contain a negatively charged

glutamic acid but an uncharged glutamine that was converted to glutamic acid by acid hydrolysis.

Species	60	61
Human β	Val	Lys
Dog β	Val	Lys
Mouse β	Val	Lys
Rabbit β	Val	Lys
1 β 4	Gln	Lys
2 β 5	Val	Lys

Sequence Homology of the β Chains - Residues 31-40

Another set of common peptides is 1 β 5 and 2 β 6 (Figure 9, Table VIIIb). Their amino acid composition were: threonine, glutamic and arginine. Since there are only three arginine residues in the β chain it is very easy to place these three amino acids into positions 38-40. It should be noted that positions 31-37 are not recovered after acid hydrolysis. Two possible explanations can be drawn. (a) Position 37 is occupied by tryptophan like other species cited here. Fragmentation might have occured due to acidic distruction of tryptophan. But 1 β 5 and 2 β 6 did not show positive Ehlich reactions on the map and this existence of tryptophan needs to be proven. (b) Two leucine and two valine residues which have bulky side chain group might cause the unproper resolution of this sequence segment.

Species	31	32	33	34	35	36	37	38	39	40
Human β	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg
Dog β	Leu	Leu	Ile	Val	Tyr	Pro	Trp	Thr	Gln	Arg
Mouse β	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg
Rabbit β	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg
1 β 5								Thr	Glx	Arg
2 β 6								Thr	Glx	Arg

Sequence Homology of the β Chains - Residues 83-87

Sequence positions 83-87 are represented by peptides 1 β 7 and 2 β 7. These two peptides have the same map location and amino acid composition (Figure 9, Table VIIIb).

Species	83	84	85	86	87
Human β	Gly-	Thr-	Phe-	Ala-	Thr
Dog β	Gly-	Thr-	Phe-	Ala-	Lys
Mouse β	Gly-	Thr-	Phe-	Ala-	Ser
Rabbit β	Gly-	Thr-	Phe-	Ala-	Lys
1 β 7	Gly	Thr	Phe	Ala	Lys
2 β 7	Gly	Thr	Phe	Ala	Lys

Sequence Homology of the β Chains - Residues 145-146

Species	145	146
Human β	Tyr-	His
Dog β	Tyr-	His
Mouse β	Tyr-	His
Rabbit β	Tyr-	His
1 β 8	Tyr	His
2 β 8	Tyr	His

An examination of common peptides 1 β 8 and 2 β 8 (Figure 9) revealed that both peptides contained tyrosine and histidine only. By homology the best places to fit them are positions 145-146 at the end of the β chain sequence.

Sequence Homology of the β Chains - Residues 88-95

Common peptides 1 β 14 and 2 β 14 (Figure 9) containing eight amino acids appear to match sequence positions 88-95 by homology and contain alanine as an extra amino acid.

Species	88	89	90	91	92	93	94	95
Human β	Leu	Ser	Gly	Leu	His	Cys	Asp	Lys
Dog β	Leu	Ser	Glu	Leu	His	Cys	Asp	Lys
Mouse β	Leu	Ser	Glu	Leu	His	Cys	Asp	Lys
Rabbit β	Leu	Ser	Glu	Leu	His	Cys	Asp	Lys
1β14	Leu	Ser	Glx	Leu	His		Asx	Lys
2β14	Leu	Ser	Glx	Leu	His		Asx	Lys

Sequence Homology of the β Chains - Residues 9-17

Species	9	10	11	12	13	14	15	16	17
Human β	Ser	Ala	Val	Thr	Ala	Leu	Trp	Gly	Lys
Dog β	Ser	Leu	Val	Ser	Gly	Leu	Trp	Gly	Lys
Mouse β	Ala	Ala	Val	Ser	Gly	Leu	Trp	Gly	Lys
Rabbit β	Ser	Ala	Val	Thr	Ala	Leu	Trp	Gly	Lys
1 β 15	Ala	Ala	Glx	Thr	Ala	Leu	Trp		Lys
2 β 10		Ala	Glx	Thr	Ala	Leu	Trp		Lys

Residues 9-17 on the β chain sequence were located in peptides 1 β 15 and 2 β 10. Homology studies indicate that one amino acid - glycine from 1 β 15 and two amino acids- alanine and glycine from 2 β 10 were missing. These missing amino acids might be destroyed or not completely released during acid hydrolysis. Position 15 in 1 β 15 and 2 β 10 is assigned tryptophan by homology and by the fact that these two peptides give positive Ehrlich reactions (Figure 9).

Sequence Homology of the 1β Chain - Peptides 1 β 6 and 1β11

In Table XI, the amino acid composition of the 1β 6 peptide represent positions 96-104 on the β chain sequence. Its peptide counterpart on the 2β map could not be located in the same position nor did it happen to be one of those peptides from the 2β map analyzed for amino acids. Therefore, it can be assumed that the sequence of amino acid

residues 96-104 on the 1β chain is different from that 2β chain.

Species	96	97	98	99	100	101	102	103	104
Human β	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg
Dog β	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Lys
Mouse β	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg
Rabbit β	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg
1 β 6	Leu	His	Val	Asx	Pro		Ala		Arg

Another indication that the 1β chain is different from 2β chain can be seen in peptide 1β 11 which represents residues 1-8 on the β chain sequence. There is also no peptide in this position in the 2β map.

Species	1	2	3	4	5	6	7	8
Human β	Val	His	Leu	Thr	Pro	Glu	Gly	Lys
Dog β	Val	His	Leu	Thr	Ala	Glu	Glu	Lys
Mouse β	Val	His	Leu	Thr	Asp	Ala	Glu	Lys
Rabbit β	Val	His	Leu	Ser	Ser	Glu	Glu	Lys
1 β 11	Val	His	Leu	Thr	Ser	Ala	Glx	Lys

Sequence Homology of the 2β Chain - Peptides 2β 9 and 2β 11

Peptides 2β 9 and 2β 11 are among those unique peptides which can be sequenced on the β chain by homology. It appears that peptide 2β 9 matches positions 133-144 on the β chain.

Species	133	134	135	136	137	138	139	140	141	142	143	144
Human β	Val	Val	Ala	Gly	Val	Ala	Asn	Ala	Leu	Ala	His	Lys
Dog β	Val	Val	Ala	Gly	Val	Ala	Asn	Ala	Leu	Ala	His	Lys
Mouse β	Val	Val	Ala	Gly	Val	Ala	Ala	Ala	Leu	Ala	His	Lys
Rabbit β	Val	Val	Ala	Gly	Val	Ala	Asn	Ala	Leu	Ala	His	Lys
2 β 9	Val	Val	Ala	Gly	Val	Ala	Asx	Ala	Leu	Ala	His	Lys

Peptide 2 β 11 represents residues 67-82 on the β chain sequence. Position 71 was assigned to glutamic acid because phenylalanine was missing from the amino acid components of 2 β 11. Instead, an extra glutamic acid residue was present. If this glutamic was not due to contamination it is reasonable to put glutamic into this position.

Species	67	68	69	70	71	72	73	74	75	76	77	78
Human β	Val-	Leu-	Gly-	Ala-	Phe-	Ser-	Asp-	Gly-	Leu-	Ala-	His-	Leu-
Dog β	Val-	Leu-	Asn-	Ser-	Phe-	Ser-	Asp-	Gly-	Leu-	Lys-	Asn-	Leu-
Mouse β	Val-	Ile-	Thr-	Ala-	Phe-	Ser-	Asp-	Gly-	Leu-	Asn-	His-	Leu-
Rabbit β	Val-	Leu-	Ala-	Ala-	Phe-	Ser-	Glu-	Gly-	Leu-	Ser-	His-	Leu-
2 β 11	Glx	Leu	Glx	Ala	Glx	Ser	Asx	Gly	Leu	Ser	His	Leu

Species	79	80	81	82
Human β	Asp-	Asn-	Leu-	Lys
Dog β	Asp-	Asn-	Leu-	Lys
Mouse β	Asp-	Asn-	Leu-	Lys
Rabbit β	Asp-	Asn-	Leu-	Lys
2 β 11	Asx	Asx	Leu	Lys

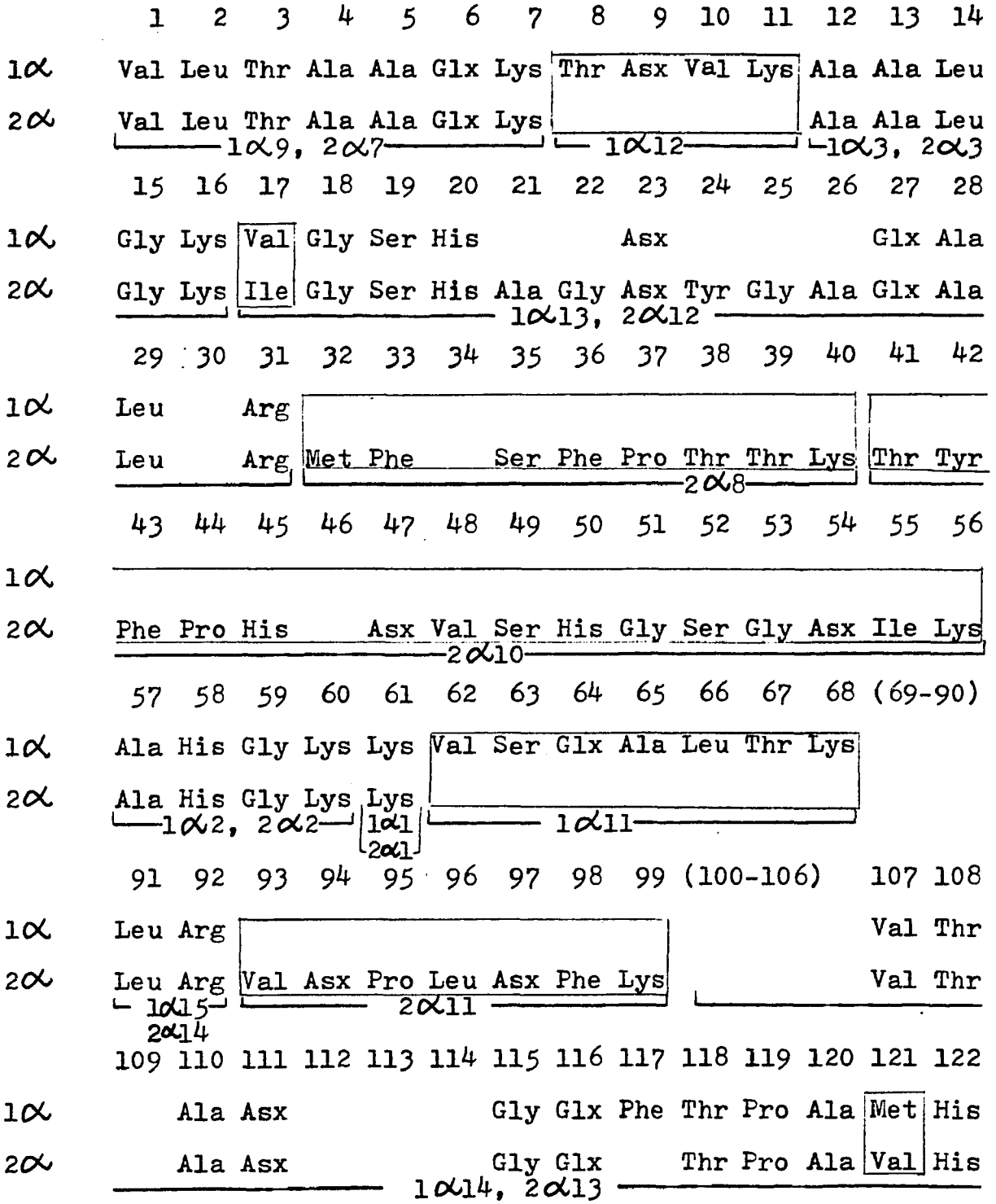
β Chain Summary

The β chains revealed the following differences:

1) Whereas the 1 β chain contains glutamic at position 60, the 2 β chain contains a valine residue.
2) Positions 1-8 and positions 96-104 located in 1 β chain are different from the same segments in the 2 β chain. This can be seen both by map position and amino acid analysis of unique peptides on the 2 β map.
3) Unique peptides 2 β 9 and 2 β 11 can be located on residues 67-82 and 133-144 in the 2 β chain respec-

tively, but the same amino acid sequence cannot be found in unique peptides on the 1β map.

Figure 11 summarizes the above and also illustrates the partial sequence of β chains of guinea pig hemoglobins 1 and 2. From these data it is probable that two β chains are significantly different.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
1α Val Leu Thr Ala Ala Glx Lys Thr Asx Val Lys Ala Ala Leu
2α Val Leu Thr Ala Ala Glx Lys Ala Ala Leu
1α9, 2α7
1α12
1α3, 2α3
15 16 17 18 19 20 21 22 23 24 25 26 27 28
1α Gly Lys Val Gly Ser His Asx Glx Ala
2α Gly Lys Ile Gly Ser His Ala Gly Asx Tyr Gly Ala Glx Ala
1α13, 2α12
29 30 31 32 33 34 35 36 37 38 39 40 41 42
1α Leu Arg
2α Leu Arg Met Phe Ser Phe Pro Thr Thr Lys Thr Tyr
2α8
43 44 45 46 47 48 49 50 51 52 53 54 55 56
1α
2α Phe Pro His Asx Val Ser His Gly Ser Gly Asx Ile Lys
2α10
57 58 59 60 61 62 63 64 65 66 67 68 (69-90)
1α Ala His Gly Lys Lys Val Ser Glx Ala Leu Thr Lys
2α Ala His Gly Lys Lys
1α2, 2α2
1α1
2α1
1α11
91 92 93 94 95 96 97 98 99 (100-106) 107 108
1α Leu Arg Val Thr
2α Leu Arg Val Asx Pro Leu Asx Phe Lys Val Thr
1α15
2α14
2α11
109 110 111 112 113 114 115 116 117 118 119 120 121 122
1α Ala Asx Gly Glx Phe Thr Pro Ala Met His
2α Ala Asx Gly Glx Thr Pro Ala Val His
1α14, 2α13

	123	124	125	126	127	(128-139)	140	141
1α	Ala	Ser	Leu	Asx	Lys		Tyr	Arg
2α	Ala	Ser	Leu	Asx	Lys		Tyr	Arg
							1α6	
							2α6	

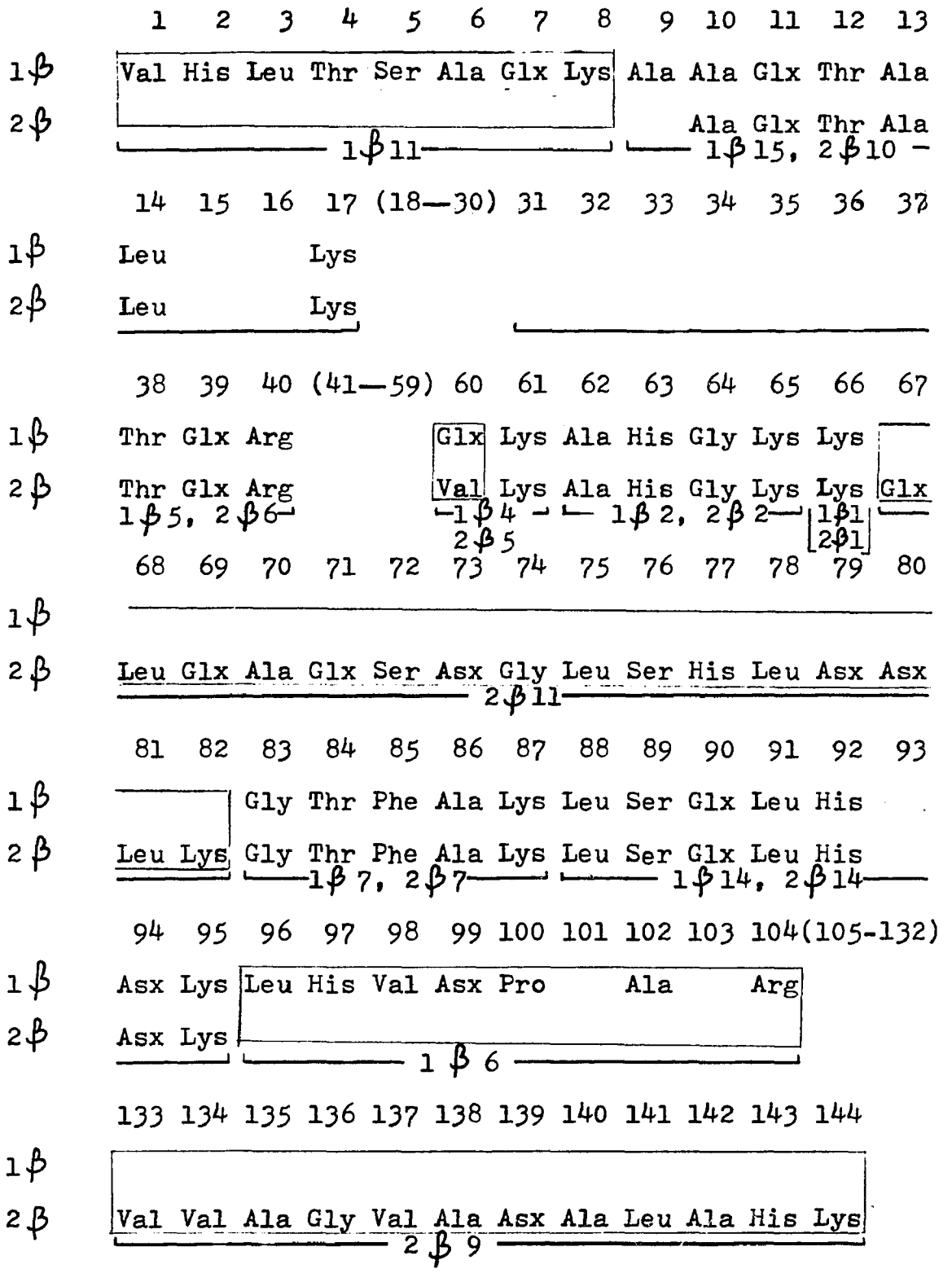
FIGURE 10

SEQUENCE BY HOMOLOGY OF THE GUINEA PIG 1α AND 2α CHAINS

□ Boxed areas indicate residue differences between the two α chains.

⌊ ⌋ Partitioned areas indicate the corresponding tryptic peptide.

( ) Bracketed numbers indicate regions on the α chains not sequenced by homology.


1 2 3 4 5 6 7 8 9 10 11 12 13
1β Val His Leu Thr Ser Ala Glx Lys Ala Ala Glx Thr Ala
2β Ala Glx Thr Ala
1β11
1β15, 2β10
14 15 16 17 (18—30) 31 32 33 34 35 36 37
1β Leu Lys
2β Leu Lys
38 39 40 (41—59) 60 61 62 63 64 65 66 67
1β Thr Glx Arg Glx Lys Ala His Gly Lys Lys
2β Thr Glx Arg Val Lys Ala His Gly Lys Lys Glx
1β5, 2β6
1β4
2β5
1β2, 2β2
1β1
2β1
68 69 70 71 72 73 74 75 76 77 78 79 80
1β
2β Leu Glx Ala Glx Ser Asx Gly Leu Ser His Leu Asx Asx
2β11
81 82 83 84 85 86 87 88 89 90 91 92 93
1β Gly Thr Phe Ala Lys Leu Ser Glx Leu His
2β Leu Lys Gly Thr Phe Ala Lys Leu Ser Glx Leu His
1β7, 2β7
1β14, 2β14
94 95 96 97 98 99 100 101 102 103 104(105-132)
1β Asx Lys Leu His Val Asx Pro Ala Arg
2β Asx Lys
1β6
133 134 135 136 137 138 139 140 141 142 143 144
1β
2β Val Val Ala Gly Val Ala Asx Ala Leu Ala His Lys
2β9

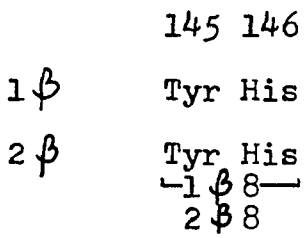


FIGURE 11

SEQUENCE BY HOMOLOGY OF THE GUINEA PIG 1β AND 2β CHAINS

[ ] Boxed areas indicate redisue differences between the two β chains.

[ ] Partitioned areas indicate the corresponding tryptic peptide.

( ) Bracketed numbers indicate regions on the β chains not sequenced by homology.

DISCUSSION

Separation of hemoglobins from red cell and marrow by the method of isoelectric focusing electrophoresis in polyacrylamide gel has revealed the presence of the same two hemoglobins insofar as their isoelectric points are identical. The distribution of the two hemoglobins in marrow was found to differ significantly from that seen in red cells, despite the fact that all hemoglobins were prepared and separated by the same procedure. Compared with other animals, the adult guinea pig is an excellent subject for studies of multiple hemoglobin synthesis because it has a relatively simple pattern of two hemoglobins. The results of the *in vivo* ^{59}Fe-labeling experiments (Table III) can be interpreted to mean that hemoglobin 2 is synthesized most actively in the youngest erythroid cells of the marrow, for the longest period of time prior to their entrance into the circulation, whereas hemoglobin 1 is most actively synthesized in older erythroid cells, which enter the circulation after only a short period of time. The time required for the specific activity of hemoglobin 2 to equal that of hemoglobin 1 is probably the maturation time of marrow erythroid cells in the guinea pig. Use of the method of dextran density gradient centrifugation which separates marrow erythroid cells according to their relative stage of development confirms the association of hemoglobin 2 with the youngest

erythroid cells and hemoglobin 1 with the oldest cells and yields direct evidence for the nonuniform distribution of hemoglobin in erythroid cell. The different relative distribution of multiple hemoglobins is also present in red cells and marrow cells of the mouse. Although the mouse marrow cells have not been separated for hemoglobin analysis, it is likely that, as in the rat and guinea pig, multiple hemoglobins of the mouse are not synthesized uniformly in marrow erythroid cells during their maturation (Figure 3; Table II).

Two polypeptide chains are considered different by using the criteria of amino acid sequence, peptide mapping and total amino acid analysis. From the chain compositions of the various hemoglobins involved, one may predict the number of structural genes. In this investigation, two different α chains and two different β chains were found. Comparisons of the total amino acid content of globin chains 1α and 2α as well as 1β and 2β revealed significant differences between the two α chains and between the two β chains (Table VIA and Table VIIA). Peptide mapping of 1α and 2α and 1β and 2β have shown the differences between them (Figure 8 and Table VIIIa; Figure 9 and Table VIIIb). Sequence studies of the tryptic peptides also reveal differences between two α chains and two β chains (Figure 10 and Figure 11). Thus two different structrual genes for α chains and two for β chains can be postulated from these studies.

Among the differences in the α chains, position 17

(Val;Ile) and position 121 (Met;Val) are occupied by different amino acids (Figure 10) and are therefore areas of potential differences. Such amino acid replacements may be the results of single point mutation i.e., GU^{U}_{C} (Val) $\longrightarrow$ AU^{U}_{C} (Ile) and AUG (Met) $\rightarrow$GUG (Val). But because of incomplete recovery of all the amino acids contained in the peptides, no reliable comparison between them can be made at this time. The remaining differences are found on some of the unique peptides. Among these, 1α12 (residues 8-11) and 1α11 (residues 62-68) could be sequenced for the α chain but no counterpart sequence can be located on the 2α chain. The same situation applies to peptides 2α10 (residues 41-56) and 2α11 (residues 93-99). These unique peptides which cannot be sequenced on the α chain may not be soluble after trypsin digestion and remain in the insoluble "core". The corresponding spots shown on the map might be fragments of peptides or incompletely digested peptides. In order for equivalent peptides in the 1α or 2α chain to be recovered, the "tryptic core" would need to be digested with chymotrypsin to reduce the peptide to smaller soluble fragments and amino acid sequence of these peptides determined.

The major differences of the two β chains are found in those unique peptides shown in Figure 11, except for position 60 in which amino acid interchange has occured between two β chains i.e., CA^{A}_{G} (Gln) $\rightarrow$$GU^{A}_{G}$ (Val). This set of assignment, however would require two base changes although most

amino acid interchanges involve a single base replacement.

The present studies demonstrate that the two hemoglobins of guinea pig which differ in their isoelectric points might be due to differences in primary structure. This was accomplished by comparing sequences, peptide maps and amino acid composition of the two α and two β chains. The possibility of post-synthesis modification is highly unlikely. Since the hemoglobins were treated with carbon monoxide before applying to the gel the possibility of differences in charge of prosthetic groups is also ruled out.

In animals, multiple hemoglobins may be due to the presence of multiple alleles of a single structural gene or to the existence of more than one structural gene for a polypeptide chain. In either case, gene duplication has occurred resulting in the production of identical proteins by more than a single gene locus. This evolutionary event is followed by sequential mutation, accounting for two or more loci that produce polypeptide chains differing by one or more amino acids, while maintaining sufficient identity for the polypeptides to be assigned as α or β. In the human, two variants of adult hemoglobin are known; the difference between them is due to differences at the β-chain locus. The genes controlling the structures of the two β chains are reported to be closely linked (Gilman et al., 1968; Winslow et al., 1966). In guinea pig, despite the

differences, two α as well as two β polypeptide chains show a great deal of homology with each other. It is postulated that the genes controlling the structure of the two α as well as two β chains are also similarly related to each other by duplication and may be closely linked.

By using dextran density gradient centrifugation technique, marrow cells can be separated according to their relative stages of maturation. Table IV shows that the more immature a population of red cells, the lower the ratio of hemoglobin 1 to hemoglobin 2. A similar conclusion has been suggested for the human (Winslow et al., 1966) in which δ chains are synthesized at a slower rate than either α or β chains of hemoglobin A or β chains of hemoglobin A_2. This can account for the small quantity of hemoglobin A_2 with respect to hemoglobin A found in normal peripheral blood. Also, as erythroid cells mature, their capacity to produce hemoglobin A_2 is lost at a greater rate than their capacity to produce hemoglobin A. In guinea pig, hemoglobin 1 synthesis may be deficient in early erythroid cells, but as cells mature hemoglobin 1 becomes the principle circulating hemoglobin component of the red cell. The mechanism for the switch from hemoglobin 2 to hemoglobin 1 production would be due to regulatory control at one of these levels:

1) cellular control: proliferation of a specific clone of cells capable of synthesizing a particular type of globin;

2) transcriptional control (gene expression): selective transcription of structural genes, efficiency of processing of globin mRNA, or efficiency of transport of globin mRNA to the cytoplasm;

3) translational control: production or activation of factor allowing selective translation of one or more particular globin mRNAs.

Since four different structural genes are involved in the two guinea pig hemoglobins, this probably makes the regulatory mechanism more complicated than two human hemoglobins which have only three structural genes. In order to determine the mechanism of regulation of hemoglobin switching, further experimental studies are needed. Another possibility is that α and β chains of a particular hemoglobin might be coordinately synthesized and coded by adjacent genes, thereby allowing the control mechanism to be simpler.

This thesis has provided evidence for the presence of multiple hemoglobins in guinea pig and that this heterogeneity is probably due to different structural genes. As has been demonstrated for the rat, guinea pig and mouse multiple hemoglobins are synthesized in a non-uniform fashion in the developing marrow erythroid cell.

A future experiment is suggested for an understanding of the possible regulatory mechanisms in non-uniformly hemoglobin biosynthesis. If younger erythroid cells of the guinea pig contain mostly globin mRNAs for hemoglobin 2 and

older erythroid cells contain mostly globin mRNAs for hemoglobin 1, this would suggest that globin mRNA for the two hemoglobins are not produced simultaneously. Regulatory control at the translational level which requires that all mRNAs are produced at the same time will then be considered unlikely. This point could be clarified by isolating globin mRNA from various bone marrow cell fractions (collected after dextran density gradient centrifugation) and assaying these mRNAs in a mRNA-dependent cell-free protein synthesizing system. After analyzing the globin product, information concerning the nature of control that causes hemoglobin switching during erythroid cell maturation would be clear and also whether this control is on the α chains, β chains or both.

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