STUDIES ON THE HETEROGENEITY OF RAT HEMOGLOBIN

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Abstract

It has been demonstrated that each individual rat has six different hemoglobins present in circulating red blood cells. The relative distribution of the hemoglobin components is constant from one animal to another, regardless of age, sex, or genetic history. This heterogeneity has been shown not to be an artifact of the methods employed for the preparation and resolution of the hemoglobin. A preparative scale method of polyacrylamide isoelectric focusing has been developed to acquire sufficient quantities of the individual components for chemical study.

All six hemoglobin fractions have the correct molecular weight and have a full complement of four molecules of heme per tetramer. Small differences have been noted in the amino acid compositions of the fractions, but they may not be real, since they are within the experimental error of the method. Variations in the amount of "surface" carboxyl groups may account for the differences in isoelectric points.

Synthesis of the components has been followed by in vivo incorporation of radioactive iron and by isolating hemoglobin from erythroid cells at different stages of development. Both techniques indicate a continuous change in the rates of synthesis of the hemoglobin fractions. The decrease in component 5 and the increase in component 4 accompanying erythroid cell maturation are the most dramatic changes observed. Under the stress conditions tested, the circulating red blood cells have the same distribution of the components as normal erythrocytes.

Circulating red blood cells of 12-day fetal rats have six hemoglobins, none of which correspond to any of the adult components. Between days 14 and 17 these fetal components are replaced by the adult components. From day 18 (3 days before birth) on, only the adult hemoglobins are present.

Introduction

It has been demonstrated that many animals, among them, the horse (Kilmartin and Clegg, 1967), goat (Huisman, et al., 1968), duck (Borgese and Bertles, 1965), mouse (Kraus, et al., 1968), as well as human (Schnek and Schroeder, 1961) have multiple hemoglobins in circulating erythrocytes. The nature of the differences among the hemoglobins within one species, both chemical and genetic, has been determined in most instances. Although it has been known for some time that the rat has multiple hemoglobins (Giri and Pillai, 1956), there is no agreement concerning the number of components.

Giri and Pillai (1956) demonstrated the presence of three hemoglobin bands, two minor and one major, in the rat by agar gel electrophoresis. Marinkovic, et al. (1967) separated rat hemoglobin into five bands by starch gel electrophoresis and found that they were genetically controlled; an inbred strain of white rats lacked the slowest moving first band, while another inbred strain of black rats lacked the third band. Most of the population of random bred rats produced all five bands, while some individual random bred rats lacked either the first or the third band.

Tobiska, et al.(1964) separated rat hemoglobin into six fractions by CMC column chromatography. They maintained that each fraction represented a distinct hemoglobin. Travnicek, et al. (1967, 1967a) resolved rat hemoglobin into four fractions on starch gel electrophoresis and into three fractions on CM-Sephadex. Hunter and Paul (1969), who studied the heterogeneity of rat hemoglobin as a function of age, reported the presence of five bands by starch gel electrophoresis in the adult.

Enoki, et al. (1966, 1966a) demonstrated the existence of six hemoglobin components by "salting out" and "alkaki denaturation" experiments, as well as by CMC column chromatography and starch gel electrophoresis. Bunn, et al. (1970) utilized polyacrylamide isoelectric focusing to resolve rat hemoglobin into seven components.

It is apparent that different groups have reported a variety of numbers of hemoglobin components despite the fact that several employed similar techniques. None of these investigators has revealed the chemical nature of the differences among the components.

Conclusive evidence is presented in this study for the existence of six erythrocyte hemoglobins in an individual rat. A method of polyacrylamide isoelectric focusing, which allows the preparation of all six fractions in a highly purified state, and in sufficient quantities for further study, is described. Also discussed are several sources for the discrepancies in the results reported in the previous research.

The possibility that the heterogeneity of rat hemoglobin is an artifact arising during the isolation procedures is discussed with regard to variations in these procedures. In addition, chemical studies on the six purified fractions are presented to demonstrate that the heterogeneity is due to differences in the primary structure of the globin chains and not to induced chemical modifications or differences due to aggregation.

Studies on the biosynthesis of the six hemoglobins are presented. The effects of phenylhydrazine induced anemia and anemia resulting from the Walker 256 carcinosarcoma on the relative proportions of the six components are discussed. In addition, the resolution of fetal rat hemoglobin by analytical isoelectric focusing is presented.

Experimental

Materials: Unless otherwise stated, all chemicals were purchased from Fisher Scientific Company and were reagent grade. CMC and DEAE cellulose were obtained from Bio-Rad. Sephadex was supplied by Pharmacia Fine Chemicals. The preparative polyacrylamide disc gel electrophoresis unit was manufactured by Canalco. Equipment for sucrose gradient isoelectric focusing, as well as Ampholines, were purchased from LKB Producter. Analytical disc gel electrophoresis and isoelectric focusing were performed in a Savant tank. Wistar rats (CFN strain) were purchased from Carworth Farms.

Preparation of hemoglobin: Rats were anesthetized by intramuscular injection of Nembutal. Blood was collected from the abdominal aorta into 0.9% sodium chloride (saline) containing a few drops of heparin as an anticoagulant. The red blood cells were washed three times by centrifugation with saline in the cold, and were then lysed at room temperature by adding three to four volumes of 10^{-4} M EDTA (ethylenediamine tetra-acetic acid) adjusted to pH 7.4. The solution was made isotonic by adding one-tenth its volume of 9% sodium chloride. At this stage as well as in all subsequent steps the hemoglobin solution was kept satuated with carbon monoxide, except for DEAE cellulose chromatography. Cell debris was removed by centrifuging at room temperature at 20,000 g for 20 minutes.

The hemoglobin was crystallized by dialysis against 5×10^{-3} M sodium phosphate, pH 7.4 overnight in the cold. The crystals were collected by centrifugation and dissolved at room temperature in 5×10^{-2} M tris (tris-

hydroxy methyl amino methane), adjusted to pH 8.6 with glycine. Final concentrations ranged from 5 to 20 mg/ml.

DEAE cellulose chromatography: A 0.9x25 cm DEAE cellulose column was equilibrated with $2.5x10^{-3}$ M sodium phosphate, pH 8.6. All buffers contained 50 mg/L potassium cyanide. The hemoglobin solution was dialyzed against $5x10^{-3}$ M sodium phosphate, pH 8.6 and about 20 mg of hemoglobin was layered on the column. The column was eluted with $2.5x10^{-3}$ M sodium phosphate, pH 8.6. At 615 ml the buffer was changed to $5x10^{-3}$ M sodium phosphate, pH 8.6. At 685 ml a linear gradient containing 75 ml each of $5x10^{-3}$ M sodium phosphate, pH 8.6 and 10^{-2} M sodium phosphate, 0.04 M sodium chloride, pH 7.9 was started. At 795 ml the second buffer of the gradient was used to wash the remaining hemoglobin fractions from the column. A flow rate of 1 ml/minute was used.

Disc gel electrophoresis: Disc gel electrophoresis was performed according to the method of Davis (1964). The separating and stacking gels were 6 cm and 0.1 cm respectively. The sample was dialyzed against the pH 8.6 tris-glycine electrode buffer overnight prior to electrophoresis. Sucrose was added to the sample in order to allow it to be layered directly over the stacking gel. Electrophoresis was conducted for 50 minutes at 4 mamp/gel at 4° C with a sample containing 0.1 gm of protein. The gels were stained with Naphthol Blue-Black (Amido-Schwartz) and were destained electrophoretically.

Preparative disc gel electrophoresis: Preparative disc gel electrophoresis was performed according to the procedure of Jovin, et al. (1964).

Analytical isoelectric focusing: Isoelectric focusing was performed in polyacrylamide gels by the method of Dale and Latner (1968) as modified by Wrigley (1968). A pH gradient from 6 to 8 was chosen. The sample

which had been exhaustively dialyzed against $5x10^{-3}$ M tris-glycine, pH 8.6, was incorporated directly into the gel prior to polymerization. Samples varying from 0.1 to 0.5 mg of hemoglobin were incorporated into gels which were 8 cm in height and 0.7 cm in diameter. Electrophoresis was carried out at 50 volts for 84 to 96 hours at 4° C. Gels were fixed with 12.5% trichloracetic acid and stained with Coomassie Brilliant Blue, according to the procedure of Chambrach, et al. (1967).

Preparative isoelectric focusing in a sucrose density gradient: Preparative isoelectric focusing was performed in a continuous sucrose gradient of 110 ml. A pH gradient of 6 to 8 was used. Electrophoresis was conducted at 600 volts for 84 to 96 hours at 4° C with a sample of 40 mg.

Scanning of analytical size gels: A Joyce-Loebl recording densitometer was used for scanning gels. Peak areas were measured in order to estimate the relative proportions of the bands.

Preparative isoelectric focusing in polyacrylamide gels: Analytical gels were scaled up 100-fold by using glass tubes 2x50 cm. A bowl with a volume of 400 ml was sealed to the top of each column to serve as a buffer compartment. The gel tubes were suspended in a wooden rack and dipped into a plexiglass buffer trough. The gel solution was bounded by 20% sucrose on the bottom and 5% sucrose on top in order to get level gel surfaces. Rubber stoppers were inserted at the bottom of each tube prior to pouring the gel and were removed after polymerization.

Forty milligrams of hemoglobin was incorporated into each gel, and electrophoresis was conducted at 500 to 600 volts for 72 to 96 hours at 4° C. After completion of electrophoresis, the gels were removed and sliced as close to the bands as possible. Material between the bands was discarded to avoid cross contamination. The gel slices were ground

in a mortar with sand as an abrasive. The hemoglobin was extracted with $5x10^{-2}$ M tris, 0.1 M sodium chloride, 10^{-4} M EDTA adjusted to pH 8.6 with hydrochloric acid. The extract was concentrated in narrow dialysis tubing by wrapping with dry Sephadex G-200. Low molecular weight contaminants were removed on a Sephadex G-75 column equilibrated with the extraction buffer.

Heme determination: Heme was measured by a modified pyridine hemochromagen procedure (Porra and Jones, 1963). An appropriate volume of sample was brought to 2.0 ml with water and 1.0 ml of pyridine and 0.5 ml of 1.0 N sodium hydroxide were added and mixed thoroughly. Enough sodium hydrosulfite was added to give a stable pink color and the optical density was read in a Beckman DU spectrophotometer at 557 mu.

Protein determination: Protein was measured by the method of Lowry, et al. (1951) using bovine serum albumin as a standard.

Molecular weight determination: Molecular weight was measured by the sedimentation equilibrium method. Each hemoglobin fraction was dialyzed against 0.10 M sodium phosphate, pH 8.0. The fractions were saturated with carbon monoxide and run at a concentration of 0.15 mg/ml in the Beckman Model E Ultracentrifuge at 12,000 rpm at 20° C overnight. A solvent density of 1.010 was used. A partial specific volume of 0.748 was assumed (Briehl, 1964). Human hemoglobin was analyzed under identical conditions for comparison.

Subunit molecular weight: Subunit molecular weights were determined by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Weber and Osborn (1969). The acrylamide stock solution contained 22.2 g of acrylamide and 0.90 g of bisacrylamide.

Preparation of hemoglobin for amino acid analysis: The hemoglobin frac-

tions were purified on a Sephadex G-75 column and dialyzed against distilled water prior to use.

Preparation of globin for amino acid analysis: To an aliquot of each fraction was added an equal volume of 10% TCA (trichloroacetic acid) in the cold. The precipitated globin was washed twice with cold 5% TCA. About 5 ml of absolute ethanol was added in the cold to dissolve the globin. Addition of about 40 ml of anhydrous ether reprecipitated the globin. After collecting the precipitates by centrifugation, the last traces of ether were removed under reduced pressure using a water aspirator. Globin was also prepared by the method of Rossi-Fanelli, et al. (1964).

Amino acid analysis: Samples of hemoglobin or globin were hydrolyzed in 6 N hydrochloric acid at 110° C. The hydrolysis tubes were evacuated with a water aspirator prior to sealing. Analyses were performed on a Beckman Model 116 Amino Acid Analyzer by the method of Moore and Stein (1963). Tryptophan was determined according to the procedure of Udenfriend and Peterson (1957) and checked by the procedure of Matsubara and Sasaki (1969). All reagents, buffers and resins used in the amino acid analyses were obtained from Beckman Instruments Co.

Determination of free carboxly groups: Free carboxly groups were determined by the method of Hoare and Koshland (1967) on denatured globin (in 8 M urea) or on undenatured hemoglobin fractions. C^{14}-glycine ethyl ester was used as the titrating agent with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as the activator. The reaction was performed at 25° C for 2 hours at pH 4.75.

Isolation of spleen hemoglobin: Spleens were homogenized in 10 volumes

of 10^{-4} M EDTA, pH 7.4 in a transi-stir homogenizer fitted with a Teflon pestle (Talboy Instrument Company). The suspension was made isotonic by addition of one-tenth of its original volume of 9% sodium chloride. The solution was kept saturated with carbon monoxide in all further steps. Cell debris was removed by centrifugation. Ammonium sulfate (Mann Research Laboratories) was added (35g/100ml) and the solution was kept for one hour in the cold. After centrifuging, ammonium sulfate was again added (35g/100ml) to the solution, which was then kept overnight in the cold. The hemoglobin precipitate was collected and dissolved in $5x10^{-2}$ M tris, adjusted to pH 8.6 with glycine. The hemoglobin was dialyzed for isoelectric focusing.

Isolation of bone marrow hemoglobin: Marrow was removed from the hind leg bones of rats and homogenized in 10 volumes of 10^{-4} M EDTA, pH7.4. The suspension was made isotonic by addition of 9% sodium chloride. Cell debris was removed by centrifugation. The solution was adjusted to pH 8.6 by addition of solid tris and kept saturated with carbon monoxide. A Sephadex G-75 column, equilibrated with $5x10^{-2}$ M tris-glycine, pH 8.6, was employed for further purification of the hemoglobin. The hemoglobin fractions were pooled, concentrated with dry Sephadex and dialyzed for isoelectric focusing.

In vivo studies with ^{59}Fe: Rats were injected intravenously with serum-bound ^{59}Fe (15 μC/rat). Normal rat serum was incubated overnight in the cold with radioactive ferric chloride (Abbott Laboratories), care being taken not to exceed the iron binding capacity of the serum (3 μg/ml). About 0.4 ml of blood was drawn from the tail of each rat at various time intervals following injection.

Red blood cells were lysed in the usual manner. The hemoglobin

solution was purified on a Sephadex G-75 column, equilibrated with 5×10^{-2} M tris-gly, pH 8.6. The purified hemoglobin was fractionated by preparative polyacrylamide isoelectric focusing. An aliquot of each fraction was taken for a heme determination by the pyridine hemochromogen method. Radioactivity was measured in a well-type radiation counter (Nuclear Chicago Company).

Phenylhydrazine induced anemia: A 1% solution of phenylhydrazine hydrochloride in 0.1 M sodium phosphate, pH 7.4 was adjusted to pH 7.4 with sodium hydroxide. Rats were injected subcutaneously with 0.5 ml of the solution on each of four successive days.

Tumor rats: Rats bearing the Walker 256 Carcinosarcoma were prepared by the subcutaneous injection of a cell suspension of the tumor prepared from a donor rat, originally supplied by the Rye Laboratories of the Sloan-Kettering Institute for Cancer Research of New York.

Separation of erythroid cells by density: Spleen cells were prepared by incubating spleen slices with hyaluronidase (Sigma Chemical Company) and collagenase (Sigma Chemical Company) for one hour at 37° C and then passed through graded wire sieves. Spleen cells were washed with saline, suspended in an equal volume of 1% albumin and separated according to density by centrifugation through a phthalate mixture (Danon and Marikovsky, 1964). The fractionated cells were washed three times with saline and hemoglobin was isolated by the ammonium sulfate precipitation method described above.

Incubation of spleen slices: Spleens were removed and rapidly transferred at cold saline, saturated with oxygen. Slices were made with a Stadie Tissue Cutter (Standard Scientific Company). The slices were suspended in Krebs-Ringer-phosphate-glucose solution (100 ml of 0.9% sodium chloride,

4 ml of 0.154 M potassium chloride, 3 ml of 0.11 M calcium chloride, 1 ml of 0.154 M monobasic potassium phosphate, 1 ml of 0.154 M magnesium sulfate, 12 ml of 0.1 M potassium phosphate, pH 7.4 and 0.24 g of glucose freshly mixed) and incubated at 37° C for one hour with serum-bound ^{59}Fe (15 μC/10g of spleen) under an atmosphere of 100% oxygen. Excess radioactivity was removed by washing the slices with cold saline. Hemoglobin was prepared by the ammonium sulfate procedure described above.

Fetal hemoglobin: Dated pregnant rats were obtained from Carworth Farms (CFN strain). Each rat was anesthetized with Nembutal and the uterus was removed. The uterine wall and the amniotic sac were cut, and the fetuses carefully removed under cold saline. After removing the placenta by cutting the umbilical cord, each fetus was washed in cold saline, and allowed to bleed into fresh cold saline by incising at several places. The red blood cell suspension, pooled for each litter, was decanted and washed three times by centrifugation in cold saline. Red blood cells were lysed with 2.0 ml of 10^{-4} M EDTA, pH 7.4. The suspension was saturated with carbon monoxide and 0.2 ml of 9% sodium chloride was added. Cell debris was removed by centrifugation. Analytical isoelectric focusing gels were prepared and stained. Only those bands which had a red color and also stained with Coomassie Blue were considered to be hemoglobins.

Results

Resolution of rat hemoglobin: Because of the relative success of column chromatography in the separation of other multiple hemoglobins (Schnek and Schroeder, 1961; Tobiska and Brada, 1964; Huisman and Dozy, 1962), the method was utilized in an attempt to separate rat hemoglobins. Noting the unusual insolubility of rat hemoglobin below pH 8.6, the anion exchanger DEAE cellulose was used. The chromatogram (figure 1) illustrates the presence of six components occurring in three pairs. Unfortunately, this separation was not reproducible nor complete. One reason for the difficulty in separating the components, is the fact that rat hemoglobin is insoluble at the pH of most of the buffers used, and crystallization occurred on the column. This insolubility also prevented the rechromatography of the components in order to remove any cross contamination.

CMC column chromatography was also attempted. Dilute hemoglobin solutions were applied, but as soon as the sample concentrated at the top of the column, crystallization occurred.

Disc gel electrophoresis also revealed the presence of six components. These bands were so closely spaced that they were not easily resolved in most gels (figure 2). Attempts at separating the components by preparative disc gel electrophoresis proved unsuccessful.

Analytical isoelectric focusing resolved rat hemoglobin into six components (figure 2). The four bands closest to the cathode were separated quite well, however, the other two bands were closely spaced. These bands could be sharpened by electrofocusing for longer periods of time, but all of the bands tended to migrate toward the cathode, and to

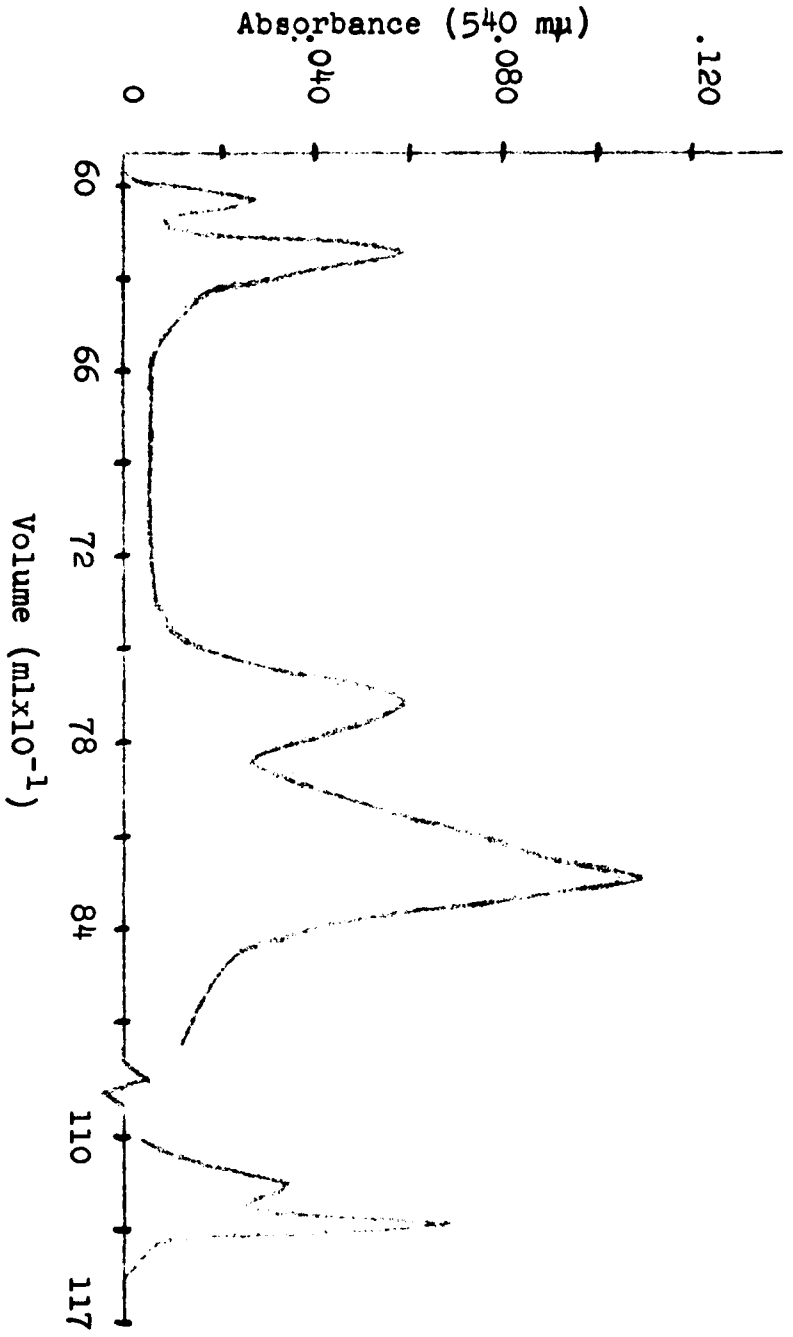

Figure 1. DEAE Cellulose Chromatogram of Rat Hemoglobin.

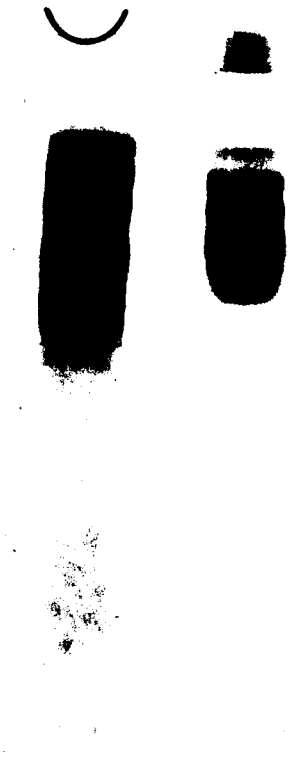

Figure 2. Isoelectric focusing, pH 6 to 8 and disc gel electrophoresis of rat hemoglobin (left to right, cathode at top).

become more closely spaced as the electrofocusing progressed. The optimum length of time could only be ascertained by observing the gels on the third and fourth days of electrofocusing.

The relative amounts of the components were determined from densitometer tracings of gels (figure 3; table 1). The values are remarkably constant from one rat to another.

Possible sources of artifacts causing the heterogeneity: It was necessary to take into account the possibility that isoelectric focusing might cause artifacts (Susor, et al., 1969). Gels were prepared by incorporating the sample only in the top half, so that the hemoglobin components started in the region of their isoelectric points (Lewin, 1969). Gels were prepared in which the sample was incorporated into the center, thus protecting it from the acidic anode and basic cathode buffers. In both instances, the same pattern of six bands was obtained. Gels were prepared using pH gradients of 5 to 8 and 3 to 10, but no new hemoglobin components were observed.

To test the possibility that crystallization of the hemoglobin might result in a fractionation of the components leading to erroneous distribution data, erythrocytes were lysed, and the total hemoglobin was not crystallized. Instead the lysate was adjusted to pH 8.6, and dialyzed for isoelectric focusing against 5×10^{-3} M tris-glycine, pH 8.6. Alternatively, the hemolysate was diluted to a concentration below 2 mg/ml, and dialyzed against 5×10^{-3} M sodium phosphate, pH 7.4, to insure that the moderately alkaline pH 8.6 buffer was not causing the multiple bands. In both instances, the normal pattern of bands was obtained.

Marinkovic, et al. (1967) have reported that the number of hemoglobins is dependent on the strain of rat chosen. This hypothesis was

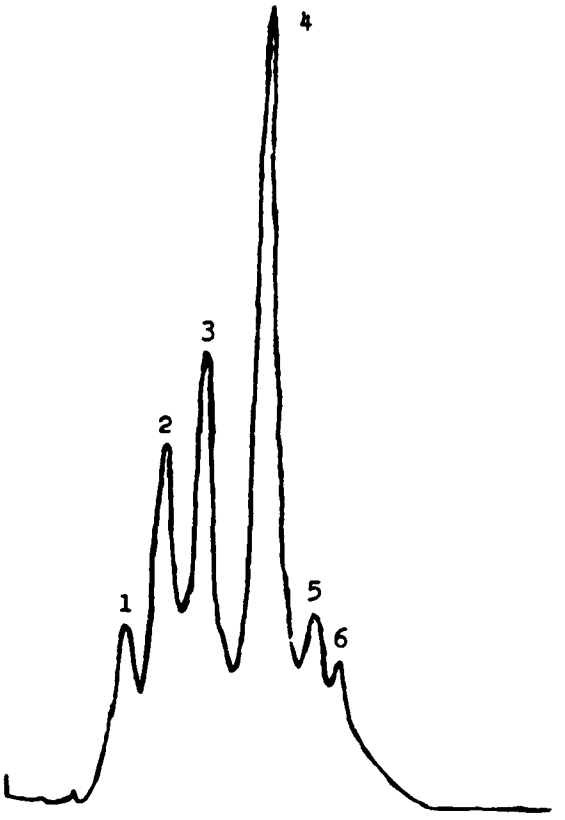

Figure 3. Densitometer tracing of rat hemoglobin resolved on an isoelectric focusing gel.

Table 1. Percent Distribution and Isoelectric Points of the Hemoglobin Components.

Fraction	% Distribution	Isoelectric Points
1	8.7 (1.0)	7.96
2	17.9 (1.3)	7.75
3	20.6 (2.5)	7.58
4	35.3 (1.8)	7.32
5	10.7 (1.6)	7.13
6	7.2 (1.7)	7.09

The distribution data are the mean values from seven gels, each representing hemoglobin isolated from an individual rat. Standard deviation is reported in parentheses.

Table 2. Strains of Rats Used for Comparison.

Strain	Source
Sprague Dawley	Carworth, N.Y.
Sherman	Camm, N.J.
Wistar	Camm, N.J.
Long Evans	Marland, N.J.
CD	Charles River, Mass.
Inbred IAC	Microbiological, Md.
Inbred LEW	Microbiological, Md.

tested by performing isoelectric focusing on the hemoglobins obtained from four different random bred, as well as two inbred (brother x sister) strains of rats (table 2). These strains were chosen, because they included those used in all other studies on rat hemoglobin. Exactly the same pattern was produced in every instance. The hemoglobin from both male and female, as well as young and old rats, yielded the same patterns. Thus, the differences between this work and others on rat hemoglobin cannot be explained on the basis of the use of genetically different rats, nor on the basis of the use of rats of varying ages or sex.

Preparation of the hemoglobin fractions for further study: Preparative polyacrylamide isoelectric focusing (figure 4) proved to be a useful technique for isolating sufficient quantities of the components needed for further study. Ten gels, each containing 40 mg of hemoglobin, could be run simultaneously. Yields of 70% were usually achieved. The purity of the components was tested by disc gel electrophoresis (figure 5). The application of large samples revealed that the first five fractions were not cross contaminated, but that fraction 6 was contaminated by fraction 5. No other proteins were observed. A second preparative isoelectric focusing step was employed to purify fraction 6. The isoelectric points were determined by measuring the pH of solutions prepared by extracting each hemoglobin containing gel slice with distilled water (table 1).

Although rat hemoglobin was separated into six components by isoelectric focusing in a sucrose density gradient (figure 6), this was not a convenient method for preparing pure hemoglobin fractions. Mixing of the bands occurred during elution, either from the bottom or from the top of the gradient.

Chemical properties of the six hemoglobins: The analytical ultracentri-

Figure 4. Rat hemoglobin fractionated on a preparative polyacrylamide isoelectric focusing gel (cathode at top).

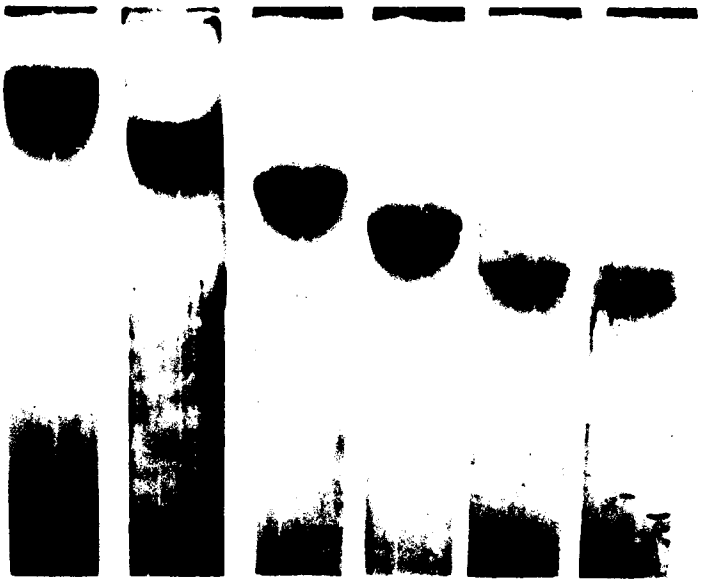

Figure 5. Disc gel electrophoresis of fractions 1 through 6 (left to right) isolated from a preparative polyacrylamide isoelectric focusing gel (cathode at top).

Figure 6. Isoelectric focusing of rat hemoglobin in a sucrose density gradient (cathode at top).

fuge was used to determine the molecular weight of each hemoglobin fraction. This was necessary in order to determine whether the observed heterogeneity of rat hemoglobin is due to differences in the state of aggregation. Frog and turtle hemoglobin, for example, have been shown to produce multiple bands on electrophoresis as a result of aggregation (Riggs, et al., 1964). Another source of heterogeneity could be due to dissociation of the tetrameric hemoglobin into monomers or dimers.

All six components have apparent molecular weights between 45,000 and 56,000 daltons (table 3). Although the molecular weight of hemoglobin should be 64,000 daltons, these lower figures are due to a rapid dissociation-association equilibrium (Schachman 1966). Human hemoglobin has an apparent molecular weight of 46,000 daltons under identical conditions (table 3).

Subunit molecular weights were determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, which causes dissociation of polymeric proteins into their monomers (Weber and Osborn, 1969). Each hemoglobin component yielded a single band by this prodedure with an Rf value corresponding to about 16,000 daltons (figure 7).

Another source of multiple components could be the loss of one or more molecules of heme during the preparation of the fractions. Heme and protein determinations on each of the fractions revealed that all six components have the same heme: protein ratio. Using the extinction coefficient of Drabkin (1942), there is an average of one mole of heme for each 17,000 $\pm$ 1,000 g (range) of protein. This agrees well with the value of 16,000 g of protein per mole of heme for all other hemoglobins studied.

Because the pyridine hemochromogen determination converts heme iron

Table 3. Molecular Weights of the Components.

Fraction	Molecular Weight
1	51,500
2	53,000
3	49,800
4	53,500
5	55,800
6	45,200
Human	46,000

Figure 7. SDS disc gel electrophoresis of hemoglobin fractions 1, 2, 3, 4, 5, and 6 and ribonuclease, cytochrome c and chymotrypsinogen A (left to right, cathode at top).

of different oxidation states to the same product (Wintrobe, 1961), the possibility remained that the components differed only in the oxidation state of their prosthetic groups. Svensson (1962) demonstrated that the isoelectric point of human hemoglobin A changed by 0.4 pH units on conversion to methemoglobin. Bunn and Drysdale (1970) have reported that conversion of rat hemoglobin to cyanmethemoglobin does not alter the pattern on polyacrylamide isoelectric focusing. This report has been confirmed in this study.

Amino Acid Analyses: The amino acid composition of each hemoglobin component was determined. Analyses were performed on both hemoglobin and globin samples. The data reported in Table IV are the averages of three determinations, except for component 6, on which one acidic and two basic amino acid determinations were performed. It is readily seen (Table 4) that the amino acid compositions of the six fractions are almost identical.

A complete amino acid analysis requires the determination of the quantity of asparagine and glutamine present in the protein. These acid amide amino acids are converted into aspartic acid and glutamic acid respectively on acid hydrolysis with the concomittant equimolar release of ammonia. Measurement of total acid amide content based on ammonia release was not feasible because of the presence of a tightly bound ammonia-releasing contaminant (either ampholines or polyacrylamide). Although these could be removed, the results were variable. Total free carboxyl groups, however, were measured for each denatured globin fraction. Like the other composition data (Table 4), the content of free acid amino acids is the same for all six fractions (Table 5).

"Surface" free carboxyl groups (pH of 4.75) were determined for each hemoglobin fraction. Fraction 1, the most basic component, had the least

Table 4: Amino Acid Composition of the Hemoglobin Components.

	1	2	3	4	5	6
Lysine	45.7 (1.3)	48.7 (1.4)	46.7 (0.9)	47.6 (1.1)	47.4 (1.0)	49.0 (1.0)
Histidine	38.0 (1.0)	39.2 (1.1)	38.3 (0.9)	38.3 (0.1)	38.4 (1.4)	39.0 (1.0)
Arginine	12.5 (0.7)	12.3 (0.3)	12.2 (0.1)	12.0 (0.1)	11.9 (0.4)	11.7 (0.6)
Aspartic Acid	57.2 (0.2)	59.0 (0.2)	60.2 (1.3)	62.0 (1.1)	62.4 (0.8)	59
Threonine	28.1 (0.1)	26.2 (1.3)	24.9 (0.3)	25.3 (0.3)	25.2 (0.8)	27
Serine	35.6 (0.6)	35.7 (1.0)	32.1 (0.7)	32.4 (0.4)	33.5 (1.0)	35
Glutamic Acid	33.5 (0.5)	33.7 (0.3)	31.9 (0.1)	31.6 (0.1)	31.9 (0.1)	35
Proline	22.6 (0.4)	22.6 (0.3)	22.5 (0.4)	22.9 (0.1)	24.2 (0.2)	23
Glycine	49.1 (0.1)	48.3 (0.4)	49.4 (0.4)	51.0 (1.2)	50.5 (0.4)	50
Alanine	71.9 (0.9)	69.7 (0.2)	70.4 (0.3)	69.2 (0.9)	69.8 (0.4)	70
Cysteine	3	4	4	4	4	4
Valine	43.9 (0.1)	45.3 (1.2)	44.7 (0.3)	45.7 (0.3)	45.7 (2.2)	47
Methionine	7.7 (0.7)	7.6 (0.6)	8.1 (0.3)	7.7 (0.1)	8.0 (0.2)	9.3
Isoleucine	12.4 (0.3)	12.5 (0.2)	12.5 (0.1)	12.4 (0.1)	12.7 (0.4)	15
Leucine	68.8 (0.2)	68.7 (0.3)	67.8 (0.9)	69.0 (0.6)	69.1 (0.7)	70
Tyrosine	11.5 (0.1)	11.9 (0.4)	12.1 (0.3)	12.1 (0.2)	12.2 (0.2)	11.9
Phenylalanine	28.2 (0.2)	28.7 (0.2)	28.7 (0.4)	29.1 (0.5)	28.6 (0.4)	30
Tryptophan	6	6	6	6	6	6

Amino Acid analyses are based on a molecular weight of 64,000 daltons.

Average deviations are given in parentheses.

Table 5: Determination of carboxyl groups by titration with C^{14}-glycine ethyl ester.

Fraction	Undenatured Hemoglobin	Denatured Globin
1	17	53
2	26	54
3	30	54
4	35	54
5	36	53
6	44	-

Values are expressed as moles of carboxyl groups per hemoglobin tetramer.

number of exposed carboxyl groups, while the other fractions had progressively greater quantities of exposed carboxyl groups (Table 5). The variation in surface carboxyl may account for the differences in isoelectric points.

Marrow and spleen hemoglobins: Hemoglobin, isolated from spleen cells, contains the same six components present in circulating red blood cells (figure 8). However, these components are present in different relative amounts (table 6). The most striking changes are the increase in component 5 and the decrease in component 4. Bone marrow hemoglobin has approximately the same distribution of components as spleen hemoglobin (figure 8, Table 6). In addition, there are small quantities of two extra bands with isoelectric points more acidic than the others (figure 8).

Since there is an incremental increase in density of erythroid cells accompanying maturation (Kovach, et al., 1967; Danon, et al., 1965), an attempt was made to fractionate spleen erythroid cells. Spleen cells were centrifuged through a mixture of dimethyl- and dibutylphthalate having specific gravity of 1.066. The fraction remaining on top represented the less dense or younger erythroid cells, while the fraction that sedimented through the phthalate represented the denser or more mature cells.

The lower fraction had a relative distribution of the hemoglobins intermediate between that of unfractionated spleen cells and that of circulating red blood cells (Table 6). This is most readily seen by looking at the ratio of components 4:5 in each instance. The 4:5 ratio is 3.2 for circulating blood cells, 0.73 for spleen cells and 1.3 for the mature spleen cell fraction. This demonstrated that there is a continuous change in the rates of synthesis of the components during matura-

Table 6: Percent Distribution of the Hemoglobin Components.

Fraction	Circulating Blood Cells	Spleen Cells	Marrow Cells	Spleen Cells	
				Top	Bottom
1	9	9	8	8	12
2	18	12	13	15	16
3	21	8	13	16	12
4	35	27	24	24	30
5	11	37	38	30	23
6	7	7	4	8	8

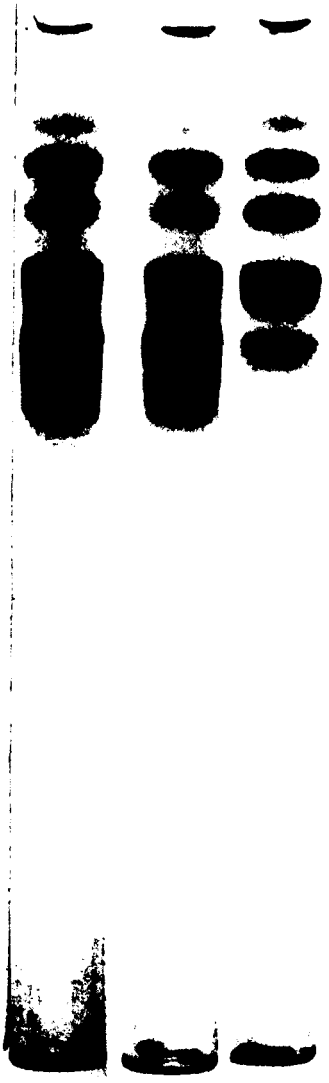

Figure 8: Isoelectric focusing of rat hemoglobin isolated from marrow, spleen, and circulating red blood cells (left to right, cathode at top).

tion, thereby eliminating the possibility of a uniform distribution of the components in all spleen cells, which changes abruptly when these cells enter the circulation. This latter possibility would necessitate a precursor-product relationship among the components.

The cells of the upper fraction contained all of the extra two acidic components (figure 9). One would expect this fraction to contain an even higher quantity of component 5 relative to component 4 than unfractionated spleen cells, since mature erythroid cells have been removed. This is not the case, for the distributions of the components of the upper fraction and the unfractionated spleen cells are about the same, that is, the 4:5 ratio is 0.73 for spleen cells and 0.80 for the less dense fraction (Table 6). One possibility for this discrepancy is that younger erythroid cells tend to be fragile (Danon, et al., 1965) and thus, the most immature cells of the upper fraction may have lysed and lost their hemoglobin.

Although it has been shown that the spleen and marrow contain the six fractions in almost identical proportions, it was necessary to prove that the mature erythroid cells released by both organs contributed the same relative quantities of each of the fractions. This was accomplished by electrofocusing hemoglobin obtained from splenectomized rat erythrocytes. Removal of the spleen leaves the bone marrow as the only source of erythrocytes. The relative distribution was found to be the same as in normal rat erythrocytes, thereby demonstrating that the spleen and marrow contribute erythrocytes with identical hemoglobin distributions to the circulating blood. The results of the above experiments demonstrate the validity of using the spleen to study erythropoiesis in the rat (Mazur, 1968). This is important, because splenic tissue is more

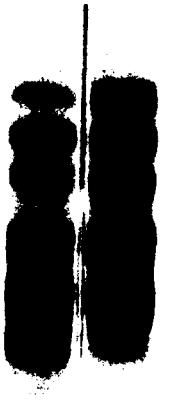

Figure 9: Isoelectric focusing of hemoglobin isolated from spleen cells fractionated according to density (top fraction on left, lower fraction on right, cathode at top).

readily available and easier to work with than marrow.

Stress rats: Administration of phenylhydrazine produces a severe anemia and thereby stimulates the erythropoietic activity of the spleen and marrow (Mazur, 1968). The circulating red blood cells yielded a hemoglobin distribution identical to that found in normal erythrocytes, in spite of the presence of a high level of less mature reticulocytes. This result contradicts that of Brada, et al. (1966), who demonstrated different relative distributions of the components of rat hemoglobin during the course of phenylhydrazine treatment.

Certain tumors, such as the Walker 256 carcinosarcome, a transplantable skin tumor, cause anemia in rats. In contrast to phenylhydrazine treatment, this anemia is due to a depression in erythropoietic activity in the marrow accompanied by a compensatory increase in activity in the spleen (Mazur, 1968). The circulating blood of tumor rats had the same relative distribution of the components found in normal rats. Thus, whether erythropoietic activity is stimulated or depressed by these methods, the circulating red blood cells had the same distribution of the hemoglobin components.

Isotopic Incorporation Studies: One of the functions of the spleen as a member of the reticuloendothelial system is to remove senescent red blood cells from the circulation. If component 5, for example, represented a hemoglobin found in senescent erythrocytes, one might expect it to accumulate in the spleen. In vitro incorporation studies with spleen slices have shown that ^{59}Fe is incorporated into all six fractions and that the specific activities of the fractions are equal, i.e., the hemoglobin components are being synthesized at rates proportional to their relative distribution. This shows that the distribution of the hemoglobins

found in splenic tissue is due entirely to de novo synthesis and not to selective removal of any of the components from the circulating blood.

In vitro studies with C^{14}-leucine, which labels globin and with C^{14}-glycine, which labels both heme and globin yielded the same specific activity for all six fractions. These results confirm the finding that each hemoglobin is synthesized at a rate proportional to its relative distribution and that there is a common pool for amino acids as well as for iron.

Synthesis of the hemoglobin components was followed by means of the in vivo incorporation of radioactive iron. Hemoglobin was prepared from blood drawn from splenectomized rats on successive days after an intravenous injection of serum bound ^{59}Fe. After one day, the specific activity of fraction 4 was more than twice that of fraction 5 in circulating red blood cells (figure 10, Table 7). However, the specific activity of fraction 5 slowly approached that of fraction 4 and by day 10 they were equal (figure 10, Table 7).

These results can be explained by assuming that the injected iron was taken up by marrow and rapidly incorporated into hemoglobin. If one were to look at the marrow at this time, one would expect to find equal specific activities for all six fractions as was found with in vitro incorporation of ^{59}Fe into spleen slices. There are cells at all stages of maturation present. However, these cells are not all released into the circulation at the same time. The erythroid cells which were released after one day were the most mature cells of the marrow at the time of injection. These cells were synthesizing fraction 4 at the highest rate relative to fraction 5. Blood drawn on later days contained cells from earlier stages of maturation at the time of injection (labeling). These

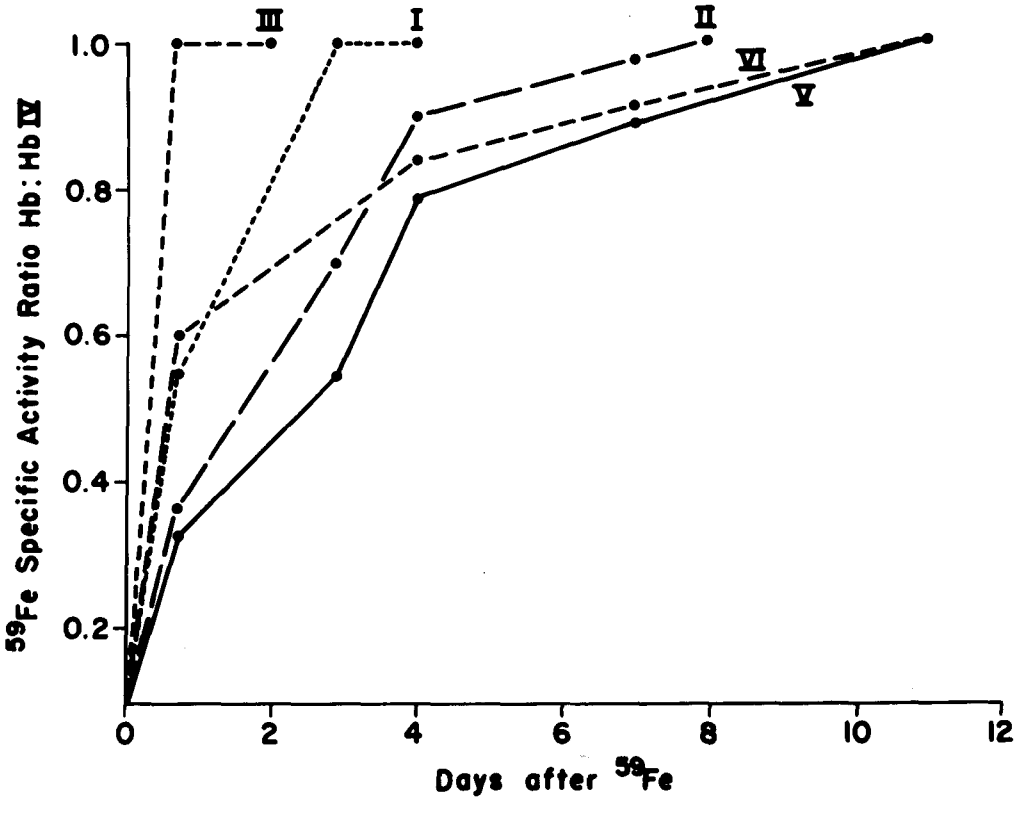

Figure 10: Release of labeled hemoglobin into circulation following an *in vivo* pulse of ^{59}Fe.

Table 7: In Vivo Incorporation of Radioactive Iron.

	cpm/μmole heme				
Fraction	17 hours	34 hours	4 days	7 days	11 days
5/4	.34±.05	.45±.07	.85±.04	.91±.01	1.02±.05

These values are the averages from three rats with the average deviations reported. The 5:4 ratio was computed for each rat and was then averaged.

cells were synthesizing progressively larger quantities of fraction 5 relative to fraction 4. Blood drawn on the tenth day contained the complete spectrum of erythroid cells present at the time of injection. In other words, it takes about ten days for the earliest erythroid cells to mature and be released into the circulating blood. The same results were obtained with normal non-splenectomized rats. Intraperitoneal injection of inorganic ^{59}Fe also gave similar results.

Fetal hemoglobin: Hemoglobin prepared from 12- and 13-day fetuses yielded six hemoglobin bands on isoelectric focusing. None of these correspond to any of the adult hemoglobins (figure 11). On day 14, the first adult hemoglobin is seen (component 2) in addition to the six embryonic hemoglobins (figure 12). Four adult hemoglobins (components 1,2,3, and 4) are present on day 15 in addition to the three most acidic embryonic hemoglobins (figure 12).

All six adult components appear on gels prepared with 16-day fetal hemoglobin (figure 13). The only non-adult hemoglobin remaining on day 17 is the most acidic fetal hemoglobin seen on day 16. Gels prepared from 18-, 19-, 20-, and 21-day fetal rats, as well as from 1-day postnatal rats are identical to those prepared from adult rats (figure 14). Figure 15 is a schematic summary of these results. There is no conclusive evidence that each of the bands is a true hemoglobin, because the quantity of fetal hemoglobin components required for such identification would be difficult to prepare, and is beyond the scope of this project.

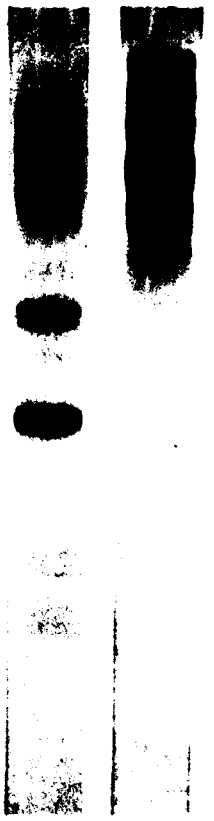

Figure 11: Isoelectric focusing of hemoglobin isolated from 12-day fetal and adult rats (left to right, cathode at top).

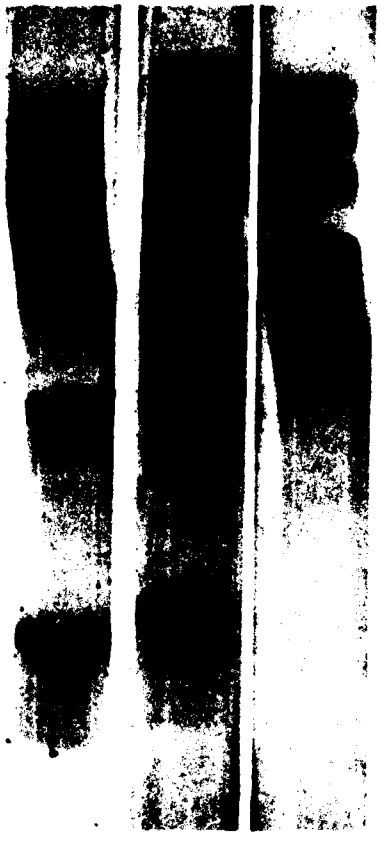

Figure 12: Isoelectric focusing of hemoglobin isolated from 14-day fetal, 15-day fetal and adult rats (left to right, cathode at top).

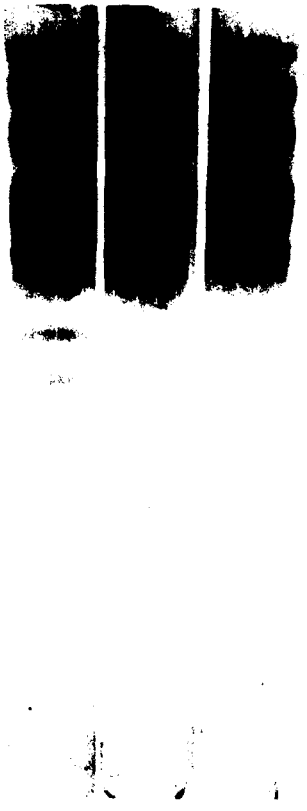

Figure 13: Isoelectric focusing of hemoglobin isolated from 16-day fetal, 17-day fetal and adult rats (left to right, cathode at top).

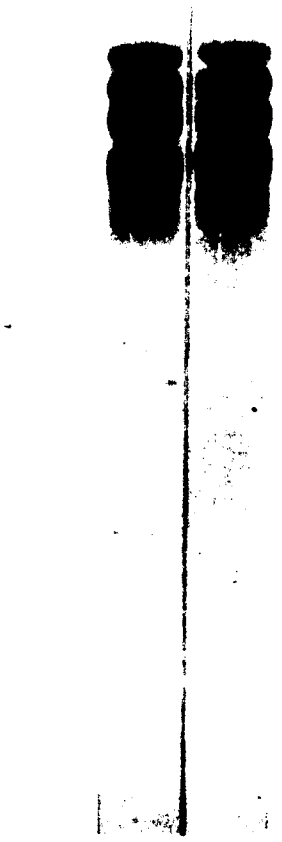

Figure 14: Isoelectric focusing of hemoglobin isolated from 18-day fetal and adult rats (left to right, cathode at top).

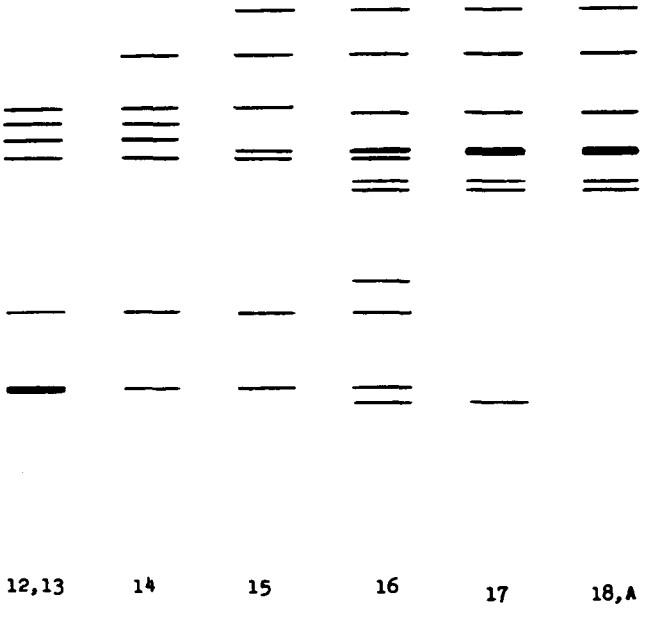

Figure 15: Schematic representation of patterns produced on isoelectric focusing gels by 12-day through 18-day fetal and adult rat hemoglobin.

DISCUSSION

Resolution of rat hemoglobin: Rat hemoglobin has been resolved into six fractions by DEAE cellulose chromatography, disc gel electrophoresis, analytical polyacrylamide isoelectric focusing, preparative polyacrylamide isoelectric focusing and isoelectric focusing in a sucrose density gradient. In all instances the relative distribution of the components is similar, with the fourth band present as the major one. It is apparent that the heterogeneity is not caused by the method employed for resolving rat hemoglobin, since these techniques are so varied. The preparation of the hemoglobin prior to its resolution by isoelectric focusing was modified, but the distribution of the components remained unaltered. Circulating red blood cells, therefore, must contain the six hemoglobins in the proportions reported in Table I.

The only explanation offered for the variety of results obtained by different researchers on the number of rat hemoglobin components is that those who reported fewer than six fractions did not get complete resolution. Bunn and Drysdale (1970,1971), who used polyacrylamide isoelectric focusing, reported the presence of seven bands. They ran their analytical gels at a high voltage (400 volts) for a short length of time (4 hours) compared with our runs at 50 volts for 3-4 days. They may have gotten oxidation of one of the components or chain separation at this high voltage. We have produced up to nine bands on isoelectric focusing at high voltage. The patterns varied among different gels run under seemingly identical conditions.

The nature of the heterogeneity: A protein may be heterogeneous in two different respects. First, an individual of the species may be isoallelic

for the protein. This is the case with human hemoglobins A and A_2. Second, different isoproteins may be present in different individuals of the species. There have been numerous human hemoglobins reported which either partially or completely replace hemoglobin A. Because the latter type of heterogeneity has not been observed in this study, even though hundreds of rats, including those from several different strains (table 2), have been tested, only the former case is discussed.

The observed heterogeneity of rat hemoglobin may be explained in several ways. All tetrameric hemoglobins studied have been shown to consist of two "α" chains and two "β" chains. The heterogeneity in human hemoglobin is due to the presence of different "β" chains; hemoglobin A is $\alpha_2\beta_2$ and A_2 is $\alpha_2\delta_2$. Rabbit hemoglobin, on the other hand, is isoallelic for the α chain, that is, $\alpha_2\beta_2$ and $\alpha^1_2\beta_2$ (Hunter and Munro, 1969). Duck hemoglobin is isoallelic for both the α and the β chain. The different isoalleles do not hybridize and therefore one finds only two hemoglobins, $\alpha_2\beta_2$ and $\alpha^1_2\beta^1_2$ (Bertles and Borgese, 1968). Humans heterzygous for hemoglobin Richmond ($\beta^{10}_{=2}$, aspN→lys) not only have $\alpha_2\beta_2$ and $\alpha_2\beta^R_2$, but also have the hybrid $\alpha_2\beta\beta^R$ tetramer (Efremov, et al., 1969).

Lactate dehydrogenase is a tetrameric protein that is composed of two different subunits that may combine in any ratio (Markert, 1963). One may have the following 5 isoenzymes: A_4, A_3B_1, A_2B_2, A_1B_3 and B_4. Cytochrome C is a monomeric heterogeneous protein, whose heterogeneity is phenotypically caused by deamidation of asparagine and glutamine residues (Flatmark and Sletten, 1968). Robinson, et al. (1970) have proposed that controlled deamidation of acid amide residues in various proteins is a biological mechanism of timing development and aging.

The multiple hemoglobins in the rat may be phenotypic in nature.

Determinations of total free carboxyl groups were performed in order to see if rat hemoglobin would fit the phenotypic model of Robinson, et al. (1970). This was not the case, but the variation in surface carboxyl groups (Table 5) suggested that the hemoglobins may differ only in their conformations. Enzymatic addition or removal of one or more N-terminal blocking groups from a single precursor, for example, could cause sufficient conformational changes to account for the variation in isoelectric points.

The similarity in amino acid composition of all six components (Table 4) supports the view that there is a single precursor hemoglobin tetramer. The fact that the same pattern of multiple hemoglobins is found throughout the population of rats tested argues against the possibility of having more than one gene for an α chain and one gene for a β chain. The existence of two α chain genes in the rabbit results in three types of individuals - those having the α^{1} gene, those having the α^{11} gene and those having both genes (Hunter and Munro, 1969). Differences in amino acid composition of the six components may be present, but may have been too small to be detected in this study. Four α chains, each differing by a single amino acid change, have been detected in horse hemoglobin (Kilmartin and Clegg, 1967). Here again, however, there is variation in the number of hemoglobins occurring in individual horses.

If the multiple hemoglobins are genotypic in nature, there must be at least three different chains, since two chains can form a maximum of five tetramers. There may be up to seven distinct chains if, for example, there is one α chain and six β chains and asymmetric tetramers of the type $\alpha_2\beta^{1}\beta^{11}$ are not allowed. The correct situation can only be determined by isolation and identification of the individual globin chains

from each of the six components.

Synthesis of the six fractions: Isolation of hemoglobin from marrow, spleen and fractionated spleen cells, clearly shows that young erythroid cells preferentially synthesize fraction 5 (Table 6). Synthesis of this component must drop off rapidly, since it comprises only 11% of the total in erythrocytes (Table 6). This is confirmed by the in vivo studies, which show that the most mature erythroid cells (those released 24 hours after the injection of ^{59}Fe) synthesize fraction 5 at only one ninth the rate of fraction 4. This value is arrived at by noting that the specific activity of fraction 5 is one third that of fraction 4 (Table 7) and the sample of circulating red blood cells used for the measurement contains a three-fold excess of fraction 4 (Table 6). The changes in the rates of synthesis of the other fractions are not as striking, but they appear to be synthesized at their greatest relative rates during the intermediate stages of erythroid cell maturation (figure 10).

A similarity exists between the mode of synthesis of rat hemoglobin and of human hemoglobin in β-thalassemia. Human hemoglobin has one major component, which consists of two α chains and two β chains. In β-thalassemia, there is a deficiency in the production of β chains. Young erythroid cells present in the bone marrow have been shown to synthesize the β chain at a normal rate (Braverman and Bank, 1969; Schwartz, 1970). As these cells mature, the rate of synthesis of the β chain decreases relative to that of the α chain. In rats, there is also a change in the relative rates of synthesis of the components as the erythroid cells mature.

Stressed rats: Under stress conditions there is a differential change in the rates of synthesis of the hemoglobins in the swiss mouse (Kraus,

et al., 1968). Besides a change in the relative proportions of the adult hemoglobins, a new component is obeerved in ducks (Borgese and Bertles, 1968) and sheep (Wilson, et al., 1966). Administration of phenylhydrazine to rats has been previously demonstrated to result in a change in the relative proportions of the erythrocyte hemoglobins (Brada and Tobiska, 1965). In contrast, phenylhydrazine treatment, as well as the presence of the Walker tumor, has revealed no change in the relative proportions of the components in this study. No explanation is offered for this contradiction.

Fetal hemoglobin: During the fetal stage, many animals possess hemoglobins which differ from those found in the adult. In sheep (Wilson, et al., 1966), cows (Babin, et al., 1966) and humans (Schroeder, et al., 1963), fetal hemoglobins comprise a major part of the total hemoglobin present at birth. Adult hemoglobins begin to appear shortly before birth, and the young animals possess only trace quantities, if any, of the fetal hemoglobins (Zuckerkandl, 1965). In addition to fetal hemoglobins, there are also human embryonic hemoglobins which disappear soon after the onset of fetal hemoglobin synthesis (Zuckerkandl, 1965).

Kovach, et al. (1967) and Fantoni, et al. (1969) studied hemoglobin synthesis in a particular strain of mouse. They demonstrated that there is one adult hemoglobin present. The fetal mouse, which has a gestation period of 21 days, has no true fetal hemoglobins, but there are three embryonic hemoglobins. Between days 10 and 15, the embryonic hemoglobins are present, while after day 15, only the adult hemoglobin is synthesized. Another strain of mouse, studied by Barker (1968), also has embryonic and adult hemoglobins, but no fetal hemoglobins. The embryonic mouse has three hemoglobins, which persist until day 16. A fourth embryonic hemo-

globin continues to be synthesized in the adult. Two additional adult hemoglobins begin to be synthesized at day 16.

There is a great similarity between the results summarized in Figure 15 and those reported for the mouse (Fantoni, et al., 1969; Kovach, et al., 1967) in that there are embryonic hemoglobins which are replaced by the adult hemoglobins well before birth. This is contrary to the development of most other animals studied, which have fetal hemoglobins that are replaced after birth by adult hemoglobins. In the 10- and 11-day fetal mouse, the three embryonic hemoglobins are synthesized in nucleated erythroid cells discharged into the peripheral blood by the blood islands of the yolk sac (Roscoe, 1966). These cells continue synthesizing the embryonic hemoglobins until about day 14. Between days 12 and 16, the liver is the major source of erythroid cells, while the spleen and the bone marrow become active erythropoietic organs at days 15 and 16, respectively. The marrow is the predominant site of erythroid cell production in the adult mouse. Adult type hemoglobin is synthesized in the liver, spleen and marrow. The same situation may apply to the rat. That is, the embryonic hemoglobins are synthesized by erythroid cells originating in the yolk sac, while the adult hemoglobins are synthesized by the other erythropoietically active organs.

There are six embryonic hemoglobins and six adult hemoglobins in the rat. This coincidence suggests that each of the embryonic hemoglobins may correspond to an adult hemoglobin. For example, the removal of an N-terminal blocking group may convert an embryonic hemoglobin into an adult hemoglobin. On the other hand, the mechanism that causes the six-fold heterogeneity of adult hemoglobin may also operate in the embryo.

Hunter and Paul (1969) have reported that there are five hemoglobins

in the adult rat. The synthesis of three of these hemoglobins begins in the 14-day fetus. The other two are synthesized as early as the tenth day of fetal life. No non-adult hemoglobins were found. The electrophoretic distribution pattern differs from that found in this study, and thus no direct comparison of results may be made.

SUMMARY

Rat hemoglobin has been resolved into six distinct components by analytical polyacrylamide isoelectric focusing. These same components are present in each individual rat, regardless of age, sex or genetic history. The heterogeneity is not due to artifacts caused by the methods emoplyed for preparation and resolution of rat hemoglobin, since altering these procedures does not change the pattern on analytical isoelectric focusing. In addition, DEAE cellulose chromatography, disc-gel electrophoresis and isoelectric focusing in a sucrose density gradient also yield six hemoglobin fractions.

Preparative polyacrylamide isoelectric focusing has yielded all six components in a highly purified state. The molecular weights determined by sedimentation equilibrium and the subunit molecular weights determined by SDS-disc gel electrophoresis are the same for all six components. In addition, each hemoglobin has the same heme:protein ratio.

Amino acid compositions are too similar to draw any conclusions about possible differences among the components. Variations in the amount of "surface" carboxyl groups may account for the differences in isoelectric points.

Young erythroid cells residing in marrow and spleen contain the six hemoglobins in relative proportions that differ from those in erythrocytes. Component 4 is the major hemoglobin in erythrocytes, whereas component 5 is the major one in spleen and marrow. Two more hemoglobin components are present in the youngest erythroid cells. Component 5 is preferentially synthesized by the youngest erythroid cells, whereas the most mature erythroid cells synthesize components 3 and 4 at the highest relative

rates. Spleen and marrow appear to be identical with respect to erythropoietic activity.

Phenylhydrazine induced anemia and anemia resulting from the Walker 256 carcinosarcoma produce no detectable change in the relative proportions of the six hemoglobins in circulating red blood cells, even though there is a high reticulocyte count.

Six embryonic hemoglobins have been detected by analytical polyacrylamide isoelectric focusing in the 12-day fetal rat. None of these components corresponds to any of the adult hemoglobins. Between days 14 and 17 the fetal components are replaced by the adult components. The normal pattern of adult hemoglobins is present from days 18 through 21, as well as in the 1-day post-natal rat.

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