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NON-UNIFORM BIOSYNTHESIS IN MARROW ERYTHROID CELLS OF MULTIPLE HEMOGLOBINS IN THE RAT

by

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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

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Abstract

NON-UNIFORM BIOSYNTHESIS IN MARROW ERYTHROID CELLS OF MULTIPLE HEMOGLOBINS IN THE RAT

by

Chien-Kuo Yeh

Advisor: Professor Abraham Mazur

Extracts of total rat erythroid cells of the bone marrow contain the same six hemoglobins (Hbs I, II, III, IV, V, and VI) as those of circulating erythrocytes, but the relative proportions of these hemoglobins differ; Hb IV is the major hemoglobin in erythrocytes, whereas Hb V is the major one in marrow. The character of the erythroid cells from bone marrow and spleen are identical in rats. Hemoglobins from these two tissues have nearly the same distribution and contain two additional more acidic hemoglobins (Hbs VII and VIII) not detected in erythrocytes. In addition, hemoglobin-containing bone marrow cells can be separated into two fractions (nucleated-rich cell and non-nucleated-rich cell) by 20% (w/w) dextran T-40. The ratios of Hb IV to Hb V are 3.61, 1.59, and 0.757 for circulating blood cells, non-nucleated marrow cells and nucleated marrow cells, respectively. This variation in the Hb IV to Hb V ratio demonstrates that there is a continuous change in the rates of synthesis of the components

during maturation.

The technique of countercurrent distribution (CCD) can be used to separate rat hemoglobin-containing bone marrow cell suspensions into fractions enriched in particular cell types. Albertsson's thin layer CCD technique is a good method for the separation of rat bone marrow cells according to their age and the two-polymer phase system does not damage the cells during the separation. Fractions from CCD separation of bone marrow cells were then analyzed by IEF. The results of this study give direct and clear evidence of non-uniform biosynthesis of multiple hemoglobins in rats. The results indicated that Hbs V, VII and VIII are associated mostly with the "youngest" erythroid cells whereas Hb IV is associated mostly with the "oldest" erythroid cells. To confirm this observation, marrow erythroid cells of rats were labelled with ^{59}Fe (in vivo) and the six hemoglobins of the circulating RBC were analyzed for specific radioactivity starting at seven hours after the ^{59}Fe injection. The specific radioactivity of Hb IV in the RBC was the highest whereas that of Hb V was the lowest. With an increase of labelling time, the specific radioactivity of Hb V reach that of Hb IV. This was substantiated by the finding that in vitro labelling by ^{14}C-leucine of reticulocyte-rich cells from phenylhydrazine-treated rats resulted in unequal specific activities. For marrow cells, in vitro labelling with ^{59}Fe or ^{14}C-leucine resulted in equal specific radioactivities for Hb I to VI, but were higher for Hb VII and VIII.

These findings are suggestive of non-uniform biosynthesis of multiple hemoglobins during erythroid cell development in the rat.

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INTRODUCTION

Studies in this laboratory have led to the separation of rat red blood cell hemoglobins (Hb) into six fractions (Hbs I, II, III, IV, V, and VI) using the extremely sensitive method of isoelectric focusing (IEF) electrophoresis in polyacrylamide gels along a pH gradient of 6.0 to 8.0 (Stein et al., 1971). The relative distribution of these six electrophoretically distinguishable hemoglobins in red blood cells (RBC) of adult rats is remarkably constant for many individuals and strains. Recently Garric et al. (1975) examined over 200 rats and found the six components, with a very relative distribution similar to ours, by both cellulose acetate electrophoresis and DEAE-cellulose chromatography. In the Stein et al. (1971) study, marrow cells and spleen cells were observed to contain not only the six distinct species as circulating red cells but also to have small amounts of two additional species (Hbs VII and VIII). Although marrow and spleen contained the same six hemoglobins as erythrocytes, their distributions in these cells differed from that of erythrocytes, especially with respect to Hbs IV and V. It was observed that, although Hb IV was the major hemoglobin in circulating red cells, Hb V was the major hemoglobin extracted from total marrow or spleen cells. Through the use of ^{59}Fe, Stein et al. (1971) found *in vivo* studies that Hb V was synthesized to the greatest extent in the "youngest" erythroid cells since its specific radio-

activity required the longest time to reach a maximum in circulating red cell, following the intravenous administration of ^{59}Fe. On the other hand, Hb IV appeared to be synthesized in the "oldest" erythroid cells since its specific radioactivity in circulating red cells was highest at the earliest time interval following ^{59}Fe administration. The specific radioactivities of the other hemoglobins varied between those for Hb IV and V.

Ellen Weiser, in this laboratory, (Doctoral thesis at CUNY, 1976) analyzed the tryptic peptides of each of the alpha and beta chains from three (Hbs III, IV, and V) of the six rat hemoglobins. She found that each of the alpha and beta chains of Hb III, IV, and V are different. Garric et al. (1975) used the urea starch gel method and found that the inbred rat red blood cell hemolyzate contained two alpha chains and three beta chains. Therefore, it is obvious that the multiple forms of rat hemoglobins are due to differences in the two different sub-units making up the protein. These multiple forms of polypeptide chains are due to genetically induced amino acid substitutions, deletions or additions. Multiple hemoglobins are synthesized in the adult mammalian bone marrow erythroid cells, and these multiple proteins are therefore excellent tools for the study of adult developing cells. The differences in distribution and in specific radioactivity of hemoglobins in the RBC and marrow cells was suggestive of the possibility that this might be due to non-uniform biosynthesis of the

multiple hemoglobins during erythroid cell development in the bone marrow.

In the present study, in vitro labelling of reticulocyte-rich cells from phenylhydrazine-treated rats with ^{14}C-leucine resulted in Hb IV having the highest specific radioactivity, and Hb V having the lowest. However, in vitro labelling of total marrow cells (precursors of the adult circulating RBC) with ^{59}Fe or ^{14}C-leucine resulted in equal specific radioactivities. It was concluded from the results of both in vivo and in vitro studies that the six hemoglobins were not being synthesized to the same extent at different stages of maturation of erythroid cells.

In order to investigate more directly the different stages at which cells synthesize these multiple hemoglobins and to confirm the data from ^{59}Fe labelling experiments in normal rats, a suitable separation technique was needed which would separate erythroid cells into fractions representing different stages of their development. If erythroid cells of different stages showed a non-uniform distribution of the multiple hemoglobins, this would strongly suggest non-uniform biosynthesis of multiple hemoglobins. In the separation techniques, subsequent collection of the cells in sufficient quantities to permit accurate biochemical characterization, was another essential prerequisite. Various attempts have been made to fractionate developing erythroblasts. Fantoni et al. (1969) utilized the fact that circulating erythroid cells of the fetal mouse increase in

maturity day by day during certain stages of embryonic development. Other investigators have isolated mouse fetal erythroid precursors from more mature erythroblasts by selective immune cytolysis, in the presence of complement and a rabbit antiserum prepared against mouse erythrocytes. By these methods erythroid cell differentiation can be studied in culture over a period of time (Cantor et al., 1972; Ramirez et al., 1975).

The physical techniques of density centrifugation (Danon et al., 1964; Hilal et al., 1964; Borsook et al., 1969), velocity sedimentation (McCool et al., 1970), sedimentation at unit gravity (Peterson et al., 1967) and countercurrent distribution (Walter et al., 1973) have been used in an attempt to separate developing erythroblasts. Walter (1969, 1975) used Albertsoon's thin layer countercurrent distribution (CCD) technique (i.e., a multiple extraction procedure in two-polymer aqueous phases) (Albertsson et al., 1962) to separate red blood cells from white blood cells and also to separate erythroblasts at different stages of development. Separation of cells by use of subtle differences in their surface properties (primarily surface charge) can often be accomplished by the suspended materials' partition in two-polymer aqueous phases (i.e., dextran and polyethylene glycol). The partition of cells by thin layer CCD is a much more sensitive indicator of cell charge properties than is cell electrophoresis. Partition is sensitive to charges located deeper into the cell membrane than is electrophoresis, which reflects only the charge at the

plane of shear. This conclusion is based on the finding of Walter et al. (1972b) that while all beef erythrocytes have the same electrophoretic mobility, these cells fall into three classes during partition, with cells having a high partition releasing far more sialic acid when treated with neuraminidase or trypsin, than do cells belonging to the other two classes. The CCD technique has been applied in the present study to characterize some of the biochemical changes that occur during erythroid cell differentiation. The results, which will be described in detail later, indicate that hemoglobin synthesis in the rat is non-uniform for the multiple hemoglobins, and is probably controlled by different genes which are activated at different stages of maturation.

The distribution of rat hemoglobins at different stages of the erythroblast may be assumed to occur by the "switch mechanism" at either the level of transcription or translation because cells at different stages synthesize different hemoglobins. The present study is focused on the development of the polymorphic hemoglobins and the relation between the possible mechanism of hemoglobin synthesis and erythropoiesis in the rat bone marrow, and to work out a new index to determine the activity of erythroid cells and a marker for the "youngest" hemoglobin-containing marrow cell. Although the cytology and cell kinetics of the maturation of the hemoglobin-containing bone marrow are characterized to a certain extent, the basic facts of the

organization, sequence of biochemical change, and enzymatic regulation during the maturation process are unknown. It is suggested that there is a gene "turn-on", "turn-off" system associated with erythroid cell maturation. In the present study of the heterogeneity of rat hemoglobin, the effects of various perturbations such as stimulation and suppression of rat marrow erythroid cells or erythropoiesis will be presented and discussed, focused on the synthesis of the six hemoglobins in rat erythroid cells. The results of this study have met objectives for separating marrow erythroid cells according to their relative stages of development and offer a new system for the study of differentiation and development in adult mammalian marrow erythroid cells on the biochemical level.

MATERIALS AND METHOD

Materials

Unless otherwise stated, all chemicals were purchased from Fisher Scientific Company and are reagent grade. DEAE cellulose was obtained from Bio-Rad Laboratories. Analytical isoelectric focusing was performed in a Savant tank. Ampholines (pH 8-6) for the isoelectric focusing experiments were obtained as special mixtures of ampholytes from LKB Instrument Company. N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylene bisacrylamide, and acrylamide were obtained from Eastman. An automatic thin-layer counter-current distribution apparatus (CCD-1200) was manufactured by Stalprodukter, Uppsala, Sweden and was obtained from Buchler Instruments, N.J., U.S.A. Sephadex and dextran T-500 (lot number 1342) and T-40 (lot number 9398) were supplied by Pharmacia Fine Chemical Company. Female Wistar rats (CFN strain) were purchased from Carworth Farms, Wilmington, Mass.

Preparation of Hemoglobin from Erythrocytes or Reticulocytes

Normal rats or phenylhydrazine-injected rats were anaesthetized by intramuscular injection of Nembutal sodium (2.5 mg/100 gm body weight) or by ether inhalation. Blood was collected from the abdominal aorta or tail vein into 0.9% sodium chloride (saline) containing heparin (50 U/ml) as an anticoagulant. The red blood cells were washed three times (by centrifugation) with saline in the cold to remove

plasma proteins and were then lysed at room temperature by adding three to four volumes of 10^{-4}M EDTA (ethylene-diaminetetraacetic acid) adjusted to pH 7.4. The solution was made isotonic by adding 1/10th its volume of 9% sodium chloride solution. At this stage, as well as in all subsequent steps, the hemoglobin solution and dialysis solutions were saturated with carbon monoxide gas to retard oxidation. Cell debris was removed by centrifugation at room temperature at 20,000 x g for 20 minutes. The hemoglobin was crystallized by dialysis against 5 x 10^{-3}M sodium phosphate solution (pH 7.4) overnight at 4°C. The crystals were collected by centrifugation and dissolved at room temperature in carbon monoxide-saturated 5 x 10^{-2}M Tris (Trishydroxymethyl amino methane) buffer, which had been adjusted previously to pH 8.6 with glycine.

Further purification was required for hemoglobin isolated from reticulocytes of phenylhydrazine-injected rats. Solutions of these hemoglobin crystals were dialyzed against Tris buffer (5 x 10^{-2}M, pH 8.6) in the cold, and passed through a column of Sephadex G-75 previously equilibrated with the same buffer. Fractions containing hemoglobin were combined, saturated with carbon monoxide and dialyzed against 5 x 10^{-3}M Tris-HCl, pH 8.6, at 4°C. After dialysis, the hemoglobin solutions were centrifuged at 20,000 x g for 20 minutes to remove any undissolved residue. 20 mg aliquots of hemoglobin were then saturated again with carbon monoxide and placed onto 2 x 8 cm DEAE

cellulose columns equilibrated with 5×10^{-3}M Tris-HCl (pH 8.6). The columns were washed with 50 ml of the same buffer to remove any impurities, and purified hemoglobin was collected by elution with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Finally, the hemoglobin solutions were prepared for isoelectric focusing by dialysis at 4°C against 5×10^{-3}M Tris-HCl (pH 8.6) in the presence of carbon monoxide.

The above procedures were performed within one week after collecting the blood samples in most cases. Otherwise, the hemoglobin was stored as a carbon monoxide-saturated solution at -15°C until the isolation procedures were initiated.

Phenylhydrazine Induced Anemia

Different dosages of a 1% solution of phenylhydrazine hydrochloride in 0.1 M sodium phosphate (adjusted to a final pH of 7.4 with sodium hydroxide) were injected subcutaneously into rats on various successive days. This stimulation of reticulocyte formation is described in detail in the "results" section.

Preparation of Rat Bone Marrow Cells

Normal or phenylhydrazine-injected adult rats weighing in the range of 200-220 gms were anaesthetized by intramuscular injection of Nembutal sodium (5 mg/100 gm body weight and as needed) and also injected intravenously with 0.1 ml heparin (1000 U/ml). After 30 minutes, 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 5 ml syringe, using for each bone 3-4 ml of chilled isotonic saline solution containing 5% (w/v) dextran (T-500) and 10 U/ml heparin. The marrow chunks were dispersed mechanically by repeated pipetting, passed through graded wire sieves, and then extruded through a No. 25 hypodermic needle. The cells were washed 3 times with phosphate buffer (pH 6.8, 0.09 M phosphate and 0.03 M NaCl) followed by centrifugation at 5°C for 20 minutes at 1000 x g after each washing. At this point the bone marrow cells are ready for preparation of hemoglobin or for separation by density gradient or countercurrent distribution.

Separation of Bone Marrow Cells by 20% (w/w) Dextran T-40

Two ml of 25% (w/w) dextran T-40 medium were placed in a Sorvall centrifuge tube (12 ml) and another two ml of 20% (w/w) dextran T-40 were layered carefully into the tube. Packed bone marrow cells (0.5 ml) suspended in six ml 10% (w/w) dextran T-40 was then layered on top of the discontinuous dextran gradient. Centrifugation was carried out at 2,000 rpm for one hour at 15°C with slow acceleration and deceleration. At the end of this process, the less dense cells were found to have moved to the top of the 20% dextran solution whereas the denser cells were found positioned between the 20% (w/w) and

25% (w/w) dextran solutions. Fine bone particles were found at the bottom of the tube. The fractionated cells were washed three times with phosphate buffer followed by centrifugation at 4°C after each wash. After this procedure the bone marrow cells are ready for preparation of hemoglobin or for further separation by countercurrent distribution.

Preparation of Hemoglobin from Rat Bone Marrow

The washed bone marrow cells were lysed at room temperature with 6 volumes of 0.001 M Tris-HCl pH 7.4, containing 0.005 M $MgCl_2$, (pH 7.4) saturated with carbon monoxide (CO). After 45 seconds of bubbling with CO, 1.5 volumes of 2.5 M sucrose containing 1.5 M KCl were added. Treatment with CO was continued for another 5 minutes. By this procedure, most of the erythroid cells were lysed while the white cells, the mitochondria, and the ribosomes of the erythroid cells remained intact. White cells, red cell stroma, and mitochondria were removed by centrifugation for 10 minutes at room temperature and 20,000 x g. The intact ribosomes and most proteins, including hemoglobin, were present in the supernatant. The ribosomes and small membrane fragments were sedimented using the Beckman L2-65 B Ultracentrifuge (60 Ti rotor) at 45,000 rpm for 120 minutes. The hemoglobin in the supernatant was purified further using Sephadex G-75 and DEAE cellulose chromatography as described earlier. Purified

hemoglobin was observed to have a $A_{280}:A_{540}$ absorbance ratio less than 3.0.

Analytical Isoelectric Focusing

Isoelectric focusing (IEF) was performed in polyacrylamide gels according to the method of Dale and Latner (1968) as modified by Wrigley (1968) and an ampholine, pH gradient from 6 to 8, was chosen. The sample which had been dialyzed exhaustively against 5×10^{-3}M Tris-glycine pH 8.6, was incorporated directly into the gel prior to polymerization. A hemoglobin sample of 0.45 to 0.55 mg was applied to each 5 x 120 mm gel. Electrophoresis was performed at 120 volts for 96 hours at 8°C. All the stock solutions used in the IEF were filtered before they were used.

Scanning of Analytical Size Gels

Gels were read at 540 nm in a densitometer immediately after electrophoresis. Peak areas were measured in order to estimate the relative proportions of the bands. The values obtained by this method were similar to those found by slicing the gels transversely and extracting the hemoglobin in each red band, followed by a spectrophotometric analysis at 410 nm.

Preparative Isoelectric Focusing in Polyacrylamide Gels

Preparative gels were poured into tubes in a volume 4 times that of analytical polyacrylamide gels (10 x 100 mm).

The electrophoresis was performed in the same apparatus (Savant Corporation) as that used for the analytical gels. Fifteen mg of hemoglobin were incorporated into each gel, and electrophoresis was performed at 120 volts for the first 24 hours and 150 volts for the next 24 to 36 hours, at 8°C. After completion of electrophoresis the following procedures were used to isolate the hemoglobin: (1) The gels were removed and sliced as close to the band as possible. Material between the bands was discarded in order to avoid cross contamination. (2) The hemoglobin of each fraction was eluted from the gel slices with 5×10^{-2}M Tris-HCl buffer (pH 8.6) containing 0.1 M NaCl and 10^{-4}M EDTA, and saturated with CO. (3) The extracts were concentrated in the cold in narrow dialysis bags wrapped in Sephadex G-200 powder and small molecules were removed by passage through a Sephadex G-75 column.

Two Polymer Aqueous Phase System for Thin Layer CCD

Buffered aqueous dextran-polyethylene glycol systems (two-phase) described by Albertsson and Baird (1962) were prepared for use in the CCD runs. In the present work, solutions containing 0.03 M NaCl and equimolar concentrations of Na_2HPO_4 and NaH_2PO_4 (0.045 M) (pH 6.8) were used as buffer. A phase system composed of 5% (w/w) dextran T-500 and 4% (w/w) polyethylene glycol 6000 was dissolved in this buffer. The two phases were allowed to equilibrate at 4°C for overnight and then were separated as top phase

(polyethylene glycol-rich solution) and bottom phase (dextran-rich solution).

Cell Separation in Countercurrent Distribution

The CCD unit is composed of two circular plexiglass plates with 120 concentric cavities. The bottom plate (or stator) cavities have a capacity of 0.664 ml. An opening above each cavity in the top plate (or rotator) is used to load cells and the phase medium. Cavities numbered 0 to 4 and 60 [illegible] received 0.45 ml each of bottom phase and [illegible] of 0.8 ml top phase and approximat[illegible] marrow cells (approximatel[illegible]). All other cavities 5 to [illegible] 0.55 ml bottom phase and 0.8 [illegible]ent distribution on this automati[illegible]eds as follows: The plates are shak[illegible]onds, during which time the phases are mixed in each cavity. Since some cells prefer the top phase and some the interface, a separation is accomplished in the loading cavities. When the phases settle (6 minutes), the top plate (rotator) rotates clockwise 3^{o} while the bottom plate (stator) remains stationary. By this procedure the top phase in each cavity is removed to the next cavity base on the stator. Those cells that were suspended in the top phase are carried to the next cavity where they are re-extracted with fresh bottom phase; those cells that were at the interface and remained behind in the bottom plate

cavity are re-extracted with fresh top phase. [illegible] is repeated 60 times in this manner. If [illegible] were used, all the cells are pipetted [illegible]

Collection of Cells from Countercurrent [illegible]

At the end of a countercurrent distribution [illegible] 1.5 ml saline was added into each cavity to [illegible] two-phase system and give rise to a single [illegible] solution. A fraction collector containing [illegible] tubes was placed over the countercurrent [illegible] rotator, and collector were "flipped over" and [illegible] cavities are simultaneously emptied into centrifuge [illegible] After the collecting tubes are removed, [illegible] then be added to the stator and rotator to wash out [illegible] residuum of cells which were not collected in the first "flip over" procedure, and the remaining cells are re-collected in the same fashion. Cells were then centrifuged at 1200 x g for 10 minutes and washed [illegible] more by centrifugation. Hemoglobin concentration was determined by the method of Drabkin (1946) at 400 nm for bone marrow hemoglobin or 540 nm for circulating cell hemoglobin on a Beckman model DU spectrophotometer. For bone marrow cells, purification of the hemoglobin before measurement of hemoglobin concentration is [illegible] sine ^{59}Fe incorporated into other cell components [illegible] for example) would affect the results. A simple way to purify marrow hemoglobin is to add one volume of [illegible] water to packed cells, frozen and thawed twice to [illegible]

(polyethylene glycol-rich solution) and bottom phase (dextran-rich solution).

Cell Separation in Countercurrent Distribution

The CCD unit is composed of two circular plexiglass plates with 120 concentric cavities. The bottom plate (or stator) cavities have a capacity of 0.664 ml. An opening above each cavity in the top plate (or rotator) is used to load cells and the phase medium. Cavities numbered 0 to 4 and 60 to 64 received 0.45 ml each of bottom phase and 0.9 ml of a mixture of 0.8 ml top phase and approximately 2.0×10^8 of rat bone marrow cells (approximately 0.1 ml packed cell volume). All other cavities 5 to 59 and 65 to 119 received 0.55 ml bottom phase and 0.8 ml top phase. Countercurrent distribution on this automatic apparatus then proceeds as follows: The plates are shaken 30 to 35 seconds, during which time the phases are mixed in each cavity. Since some cells prefer the top phase and some the interface, a separation is accomplished in the loading cavities. When the phases settle (6 minutes), the top plate (rotator) rotates clockwise 3° while the bottom plate (stator) remains stationary. By this procedure the top phase in each cavity is removed to the next cavity base on the stator. Those cells that were suspended in the top phase are carried to the next cavity where they are re-extracted with fresh bottom phase; those cells that were at the interface and remained behind in the bottom plate

cavity are re-extracted with fresh top phase. The cycle is repeated 60 times in this manner. If 120 transfers were used, all the cells are pipetted into cavities 0 to 9.

Collection of Cells from Countercurrent Distribution

At the end of a countercurrent distribution run, 1.5 ml saline was added into each cavity to break the two-phase system and give rise to a single, less viscous solution. A fraction collector containing 120 plastic tubes was placed over the countercurrent plates, the stator, rotator, and collector were "flipped over" and the 120 cavities are simultaneously emptied into centrifuge tubes. After the collecting tubes are removed, 2 ml of saline can then be added to the stator and rotator to wash out the residuum of cells which were not collected in the first "flip over" procedure, and the remaining solution is re-collected in the same fashion. Cells were then centrifuged at 1200 x g for 10 minutes and washed twice more by centrifugation. Hemoglobin concentration was determined by the method of Drabkin (1942) at 410 nm for bone marrow hemoglobin or 540 nm for circulating red cell hemoglobin on a Beckman model DU spectrophotometer. For bone marrow cells, purification of the hemoglobin before measurement of hemoglobin concentration is necessary sine ^{59}Fe incorporated into other cell components (ferritin for example) would affect the results. A simple way to purify marrow hemoglobin is to add one volume of ice cold water to packed cells, frozen and thawed twice to assure

complete lysis. The hemoglobin crystals and stroma are then washed twice with two volumes of ice cold water. The hemoglobin crystals are dissolved in Drabkin solution (200 mg of $K_3Fe(CN)_6$, 50 mg of KCN and 1,000 mg of $NaHCO_3$ to 1 liter of distilled water) at room temperature with vigorous shaking. Finally, the stromal residue is removed by high-speed centrifugation (20,000 x g).

Incubation of Bone Marrow Cells or Reticulocyte-Rich Blood Cells with Radioactive Precursors (^{59}Fe or L-^{14}C-leucine)

Washed normal bone marrow cells or phenylhydrazine-stimulated reticulocyte-rich blood cells were incubated at 37°C for various times in different experiments in a medium prepared essentially according to Borsook et al. (1957) but containing either serum-bound ^{59}Fe (ferrous citrate, Abbott laboratories) or L-^{14}C-leucine (New England Nuclear); (for amounts, see description of individual experiments). Following incubation, either chilled non-labelled serum-bound iron or unlabelled leucine was added and the cells were washed 5 times with cold saline.

X-Irradiated Rats

Irradiation was performed by Dr. Eric Hahn of the Sloan-Kettering Research Institute. X-irradiation was delivered by a General Maxitron 300 therapy unit operated at 300 kVp and 20 mA. The HVL was 1.65 mm Cu, and the distance from target to sample was 30 cm. The dose rate to the splenectomized rat was 420 rads/min. A total of 900 rads (lethal dose) was delivered to each rat. Three

days after the irradiation, the bone marrow cells were isolated and put into CCD.

Serum-Bound ^{59}Fe Injection

Normal rat serum was incubated overnight in the cold with radioactive ferric chloride (Abbott Laboratories) with the pH adjusted to 7.4. Rats were injected intravenously with 0.5 ml of serum-bound ^{59}Fe (6×10^6 cpm/rat) and the animals sacrificed 4 hours after ^{59}Fe administration. Bone marrow cells and red blood cells were then isolated for countercurrent distribution studies. In some experiments 0.3 - 0.4 ml of red blood cells were drawn from the tail of each rat at various time intervals following injection, for the *in vivo* studies with ^{59}Fe.

Microscopic Counting of Cells

Cells were diluted with 0.9 NaCl (pH 7.0) and counted under the microscope.

Protein Determination

Total protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Hemoglobin concentrations were determined spectrophotometrically at 540 nm or at 410 nm.

DNA and RNA Determinations

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) could be extracted by hot ($90^{\circ}C$) 5% TCA and hot ($70^{\circ}C$) 2% perchloric acid (PCA) after treatment of the cell lysate

with 10% TCA. The DNA content of the cell was determined by the Giles and Myers' modification (1965) of the Dische diphenylamine reaction, using calf thymus DNA as a standard. RNA was determined by the orcinaol method (Dische et al., 1955) using adenosine as a standard.

Determination of Radioactivity

Aliquots of lysates containing ^{59}Fe were pipetted into tubes and counted in a well-type crystal scintillation counter (Nuclear Chicago Company). The lysed cells, containing ^{14}C, were precipitated with 10% trichloroacetic acid (TCA). After standing for several hours the precipitate was redissolved with 1 N NaOH and then reprecipitated with a large volume of 7% TCA and collected by centrifugation. The supernatant was discarded. This precipitate was washed successively with (1) 10% TCA at 80 - 90°C for 30 minutes, (2) acetone (0.5% HCl) at -20°C, (3) ether:ethanol (1:1) at 35°C for 30 minutes and (4) ether for 15 minutes (two such washings). The precipitate was air dried and dissolved in a minimum volume of saline. The solution was ready for protein determination and radioactivity determination.

Ten ml of scintillation fluid (Bray's solution) (Bray, 1960) were mixed with radioactive (^{14}C) protein solution in the counting vials. The ^{14}C samples were counted in a Beckman LS-150 liquid scintillation counter.

Polycythemic Rats and Starved Rats

This mehtod is described in the "results" section.

RESULTS

Marrow Hemoglobins

Hemoglobin, isolated from bone marrow cells, contains the same six components that are present in circulating red blood cells (Figure 1). However, these components are present in different relative amounts (Table 1.). The most striking changes from hemoglobin of marrow cells are the increases in relative amounts of Hb IV and the decreases in Hb V in red cells. In addition, in marrow hemoglobin there are small quantities of two extra bands with isoelectric points more acidic than the others (Hbs VII and VIII).

"Young" and "old" erythroid cells can be separated by density gradient centrifugation because the "age" of the erythroid cell is generally determined by the relative size of its nucleus. The larger the volume of the nucleus, as compared with the cytoplasm, the lighter the cell. Therefore, there is an incremental increase in density of erythroid cells accompanying maturation (Kovack, et al., 1967; Danon et al., 1965; Hilal et al., 1964). Marrow cells were suspended in 10% (w/w) dextran T-40 and centrifuged through a 20% (w/w) T-40 dextran solution. The fraction remaining between suspending solution and 20% (w/w) dextran T-40 solution represented the less dense or younger nucleated erythroid cells, while the fraction that sedimented through the 20% (w/w) dextran T-40 solution represented

the denser or more mature marrow cells and trapped circulating erythrocytes.

In Table 1, less dense bone marrow cells, total bone marrow cells, denser bone marrow cells and red blood cells correspond to the "youngest", "young", "near mature", and "mature" cells respectively in the erythroid cell series. This is seen most readily by looking at the ratio of components Hb IV/Hb V in each instance. The Hb IV/Hb V ratio is 0.757 for "youngest" (less dense) erythroid cells, 0.805 for "young" (total bone marrow cells) erythroid cells, 1.59 for "near mature" erythroid cells (denser marrow cells) and 3.61 for "mature" cells (circulating blood cells). Thus a continuous change in the distribution of the components during maturation is demonstrated. These results eliminate the possibility of a uniform distribution of the components in all marrow cells and suggest that the distribution changes abruptly when these cells enter the circulation as cells which no longer are capable of hemoglobin synthesis. Studies by Stein et al. (1971) suggested similar findings except that spleen cells and a mixture of dimethyl and dibutylphthalates (specific gravity of 1.066) were used for separation of cells. The present work was more effective in the separation of less dense cells than that of Stein et al. (1971) since Hb IV/Hb V is 0.73 for total spleen cells and 0.80 for less dense fraction of spleen cells in the work of Stein et al. (1971). The reasons for this may be that the

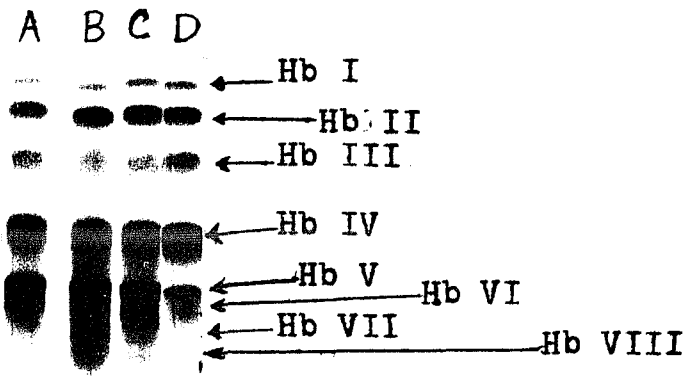


Figure 1. Rat hemoglobin fractionated on an analytical polyacrylamide isoelectric focusing gel between pH 6 to 8 (Cathode at top).

A = the denser bone marrow cell hemoglobin

B = the less dense bone marrow cell hemoglobin

C = the total bone marrow cell hemoglobin

D = erythrocyte hemoglobin

Table I. Percent distribution and isoelectric point of the hemoglobin components.

Hbs[1]	IEP[2]	Percent Distribution			
		Circulating RBC	Less Dense BMC	Denser BMC	Total BMC
I	7.96	5.8±1.2	3.4±0.3	5.3±0.3	4.6±1.7
II	7.75	16.6±1.0	14.2±1.9	15.8±0.4	14.3±2.1
III	7.58	19.5±1.6	7.6±2.4	13.1±1.9	10.5±0.9
IV	7.32	40.1±2.5	16.8±2.2	30.3±1.9	24.0±1.2
V	7.13	11.1±0.5	22.2±2.0	19.0±2.5	29.8±2.3
VI	7.09	6.7±1.0	15.6±1.3	16.5±1.1	7.9±1.1
VII	6.90	—	12.6±1.4	—	5.3±1.1
VIII	6.78	—	7.1±1.0	—	3.7±0.9

The values represent percentage distribution calculated from areas of densitometric tracings of analytical isoelectric focusing polyacrylamide gels. The distribution data are the mean values from 3 or 4 gels, each representing hemoglobin isolated from an individual rat or from a group or rats. Standard diviation is reported. Hemoglobin bands were cut from the IEF gel (10 x 100 mm) and placed in test tubes into which one ml of distilled water was added. The hemoglobin was allowed to elute overnight in the refrigerator. The pH's of the eluates were measured with a semi-micro combination pH electrode at 22°C.

1. Hbs = Hemoglobin Fraction
2. IEP = Isoelectric Point

dextran which is a physiologically inert substance disperses the cells (Bernstein et al., 1962) and preparation of bone marrow cells do not follow incubation of marrow with hyaluronidase and collagenase at 37°C for 1 hour as in the earlier method. Lysis of some of the cells and loss of some components of cell membrane during incubation is evident. Therefore, the fragile "youngest" erythroid cells were not damaged greatly in bone marrow cell suspension preparations and in the dextran solution. This is important, because further studies of the fractionation of young erythroid cells involved dextran solution. Obviously, bone marrow cells should be used in future experiments even if splenic tissue is more readily available and more easily obtained in quantity than the marrow.

Countercurrent Distribution Patterns of Bone Marrow Cells and of Red Blood Cells from Normal and from Phenylhydrazine-Injected Rats

To prove directly the reality of non-uniform distribution, and therefore of biosynthesis, of rat hemoglobins in marrow erythroid cells, the separation of erythroid cells representing different stages of development was accomplished by partition in aqueous two-polymer phase systems in the present study. This method (Albertsson et al., 1962) is based on the partition technique of thin layer countercurrent cistribution. The partition of cells in dextran-polyethylene glycol phases is determined mainly by surface

charge and has been shown to measure charge-associated properties much deeper into the membrane than does cell electrophoresis (Walter et al., 1972b).

Four typical countercurrent distribution curves are shown in Figure 2A - D of circulating red blood cells (Figure 2A, 2C) and of hemoglobin-containing bone marrow (Figure 2B, 2D) from normal (Figure 2A, 2B) and from phenylhydrazine-injected (Figure 2C, 2D) rats. Rats were injected intravenously with serum-bound ^{59}Fe (6×10^6 cpm/rat) 2 to 4 hours prior to collection of the marrow or blood. Lajtha (1966) found that the least mature erythroid cells incorporate ^{59}Fe more actively than the more mature cells. Therefore, in vivo administration of serum-bound ^{59}Fe two to four hours prior to collection of the marrow or the RBC enabled us to identify the relative distribution of "young" and "old" erythroid cells in the CCD experiments.

For normal rat circulating red cells (Figure 2A), the distribution of hemoglobin in various cavities showed a single optical absorption peak for hemoglobin (540 nm) and radioactivity (cpm for ^{59}Fe) at cavity #45, with a very low concentration of hemoglobin at cavities #25. There is higher specific radioactivity at both ends of the peak than that observed in the middle of the peak. This is explained by the observation of Walter (1966) that the cells at the right side of the peak are the youngest erythrocytes, and that the cells at the left side of the peak are the reticulocytes.

For rats treated with phenylhydrazine, a chemical which accelerates red cell destruction and the appearance of excessive numbers of reticulocytes in the blood stream, the distribution of hemoglobin of these circulatory reticulocytes (Figure 2C) showed a peak of radioactivity at cavity #41 with a distribution of hemoglobin as low as cavity #15. The specific radioactivity of cells on the left side of the peak is higher than that of cells on the right side (shown in Figure 2C).

These results indicate that reticulocytes at the extreme left (i.e., #15) end were less mature than those in the cavities at the right side (i.e., #55). Therefore, the erythrocytes were distributed at the right side of the extraction train, whereas reticulocytes are distributed according to maturity from the left side to the right side and they are separated in earlier cavities.

For normal rat bone marrow cells which were separated in a similar manner, two peaks were obtained as measured by hemoglobin absorption of the hemolysates (Figure 2B). The larger peak occurs at cavity #39 and the smaller one at cavity #29. The major peak of radioactivity was found to be associated with cavity #25 and the highest specific radioactivity was shown at the extreme end of the left side of the distribution curve (Figure 2B). The larger peak corresponds in position to the youngest reticulocytes that appear first in the peripheral circulation. The distribution of hemoglobin-containing bone marrow cells in rats pre-treated

Figure 2A. Countercurrent distribution pattern of erythrocytes from normal rats; •—•—• = absorbance at 540 nm, o--o--o-- = radioactivity (cpm), Δ—Δ—Δ—Δ = specific radioactivity (cpm/O.D. at 540 nm). Normal adult rat was injected intravenously with serum-bound ^{59}Fe four hours prior to the collection of RBC followed by the CCD procedure. The two-polymer phase system is described in the "Materials and Method" section, 60 transfers were completed at 4 - 5°C.

(Diagram is on the next page.)

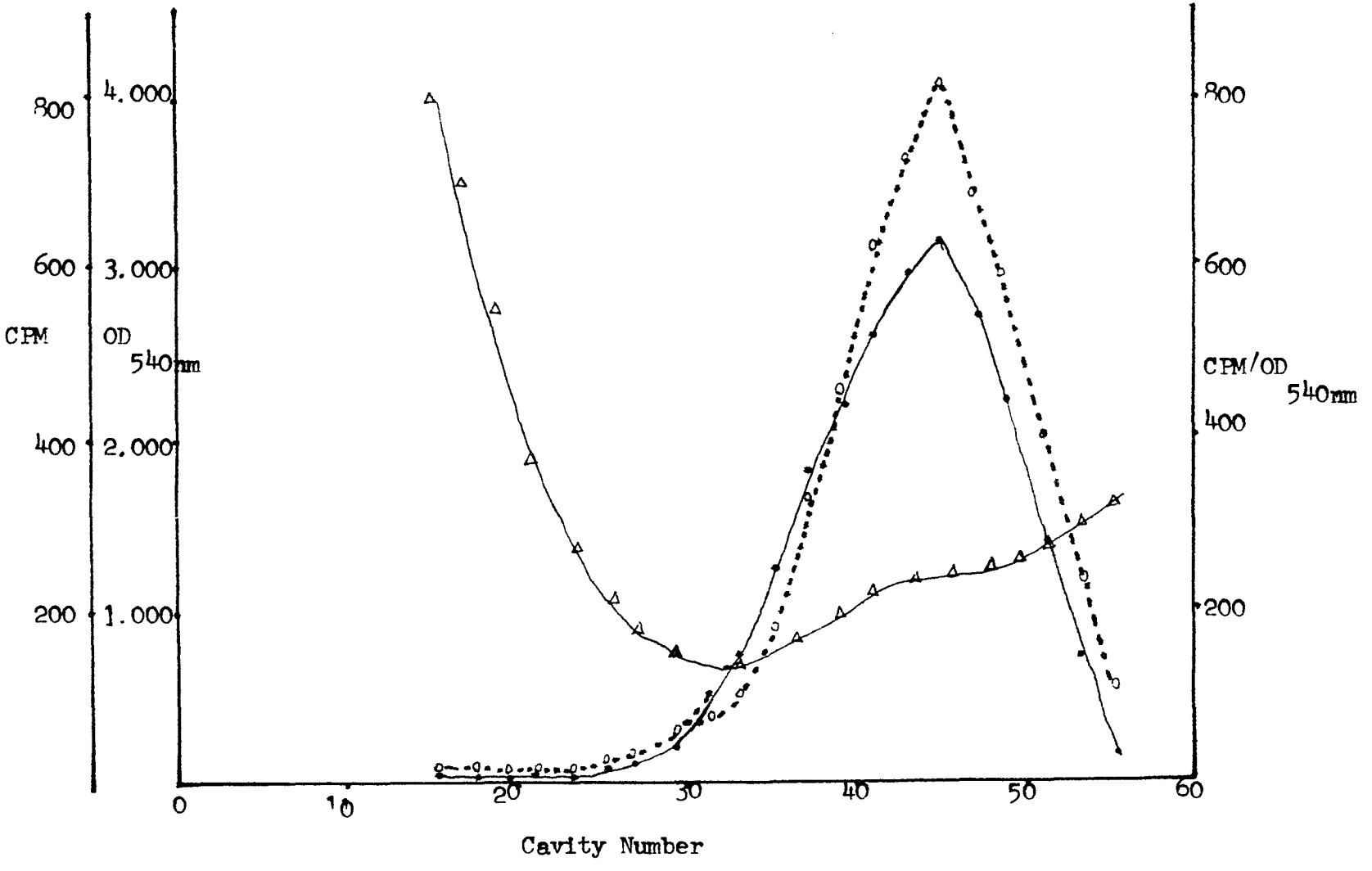

800
600
400
200
CPM
4.000
3.000
2.000
1.000
OD
540nm
0
10
20
30
40
50
60
Cavity Number
800
600
400
200
CPM/OD
540nm

Figure 2B. Countercurrent distribution pattern of hemoglobin-containing bone marrow cells from normal rats; •—•—•—• = absorbance at 410 nm, o- - -o- - -o- - -o = radioactivity, Δ—Δ—Δ—Δ = specific radioactivity (cpm/O.D. at 410 nm). Three normal adult rats were injected intravenously with serum-bound ^{59}Fe two hours prior to removal of bone marrow. Cells were partitioned by thin layer CCD into 60 fractions. Extracts of each fraction were assayed for ^{59}Fe and hemoglobin content.

(Diagram is on the next page.)

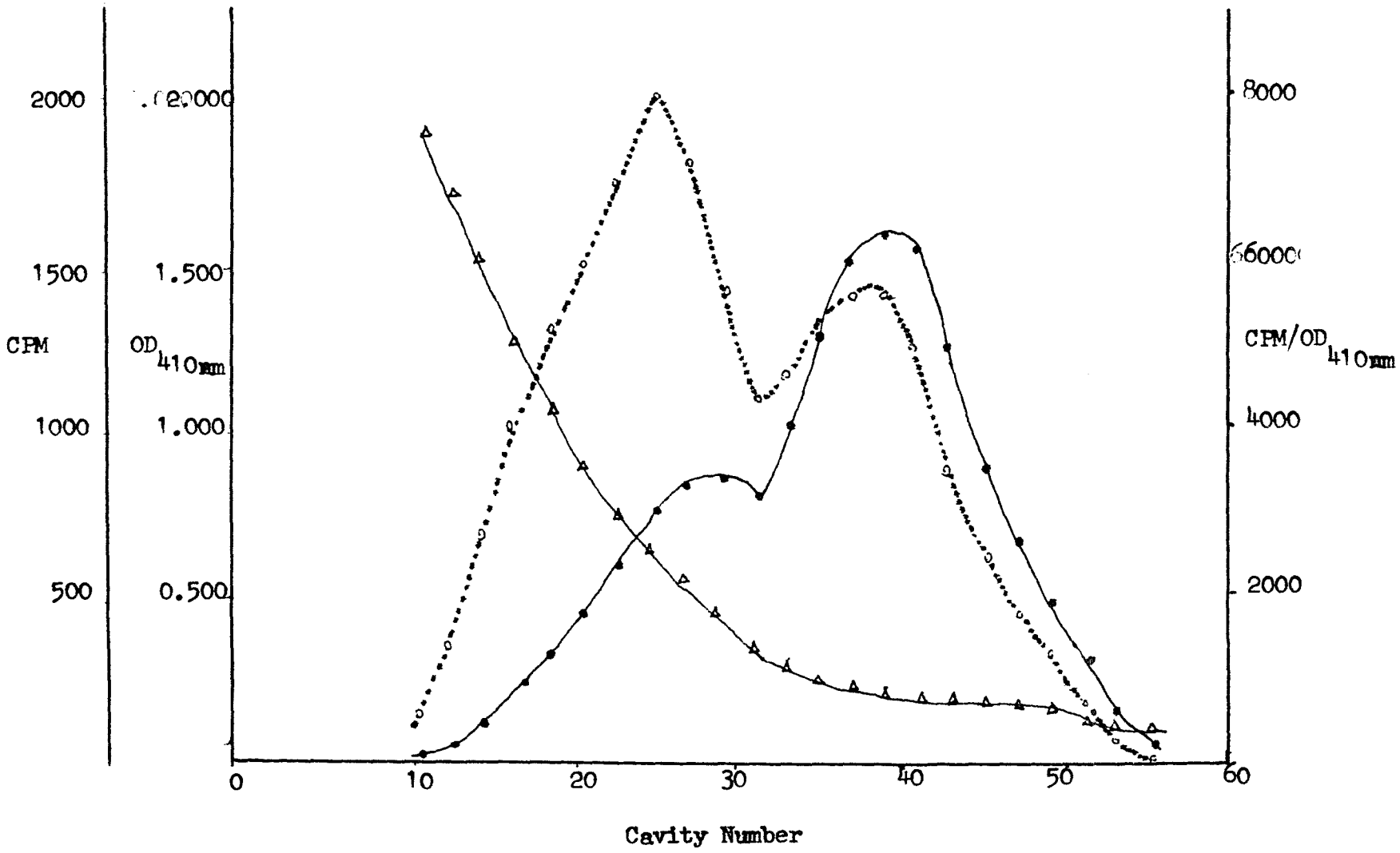

2000
1500
1000
500
CPM
.020000
1.500
1.000
0.500
OD410nm
8000
6000
4000
2000
CPM/OD410nm
0
10
20
30
40
50
60
Cavity Number

Figure 2C. Countercurrent distribution pattern of circulating red cells from phenylhydrazine-injected rats; •—•—•—• = absorbance at 540 nm, o- - - o- - -o = radioactivity, Δ——▷——▲ = specific radioactivity (cpm/O.D. at 540 nm). Rats were injected with 0.5 ml phenylhydrazine (1%) on each of 4 successive days. Serum bound ^{59}Fe was injected on the 6th day. 2 hours later, the rats' red blood cells were subjected to countercurrent distribution (as in Figure 2A). (Diagram is on the next page).

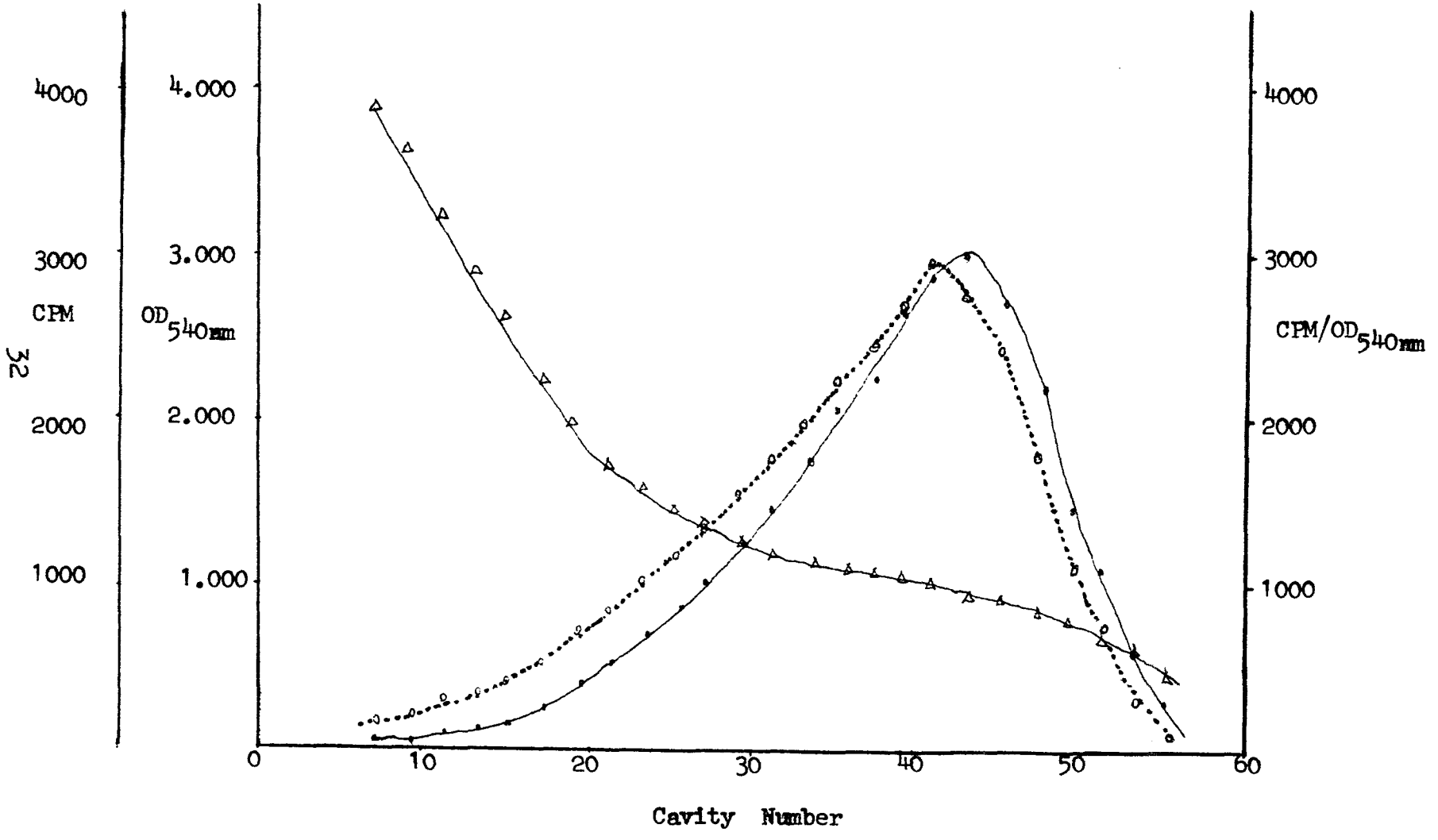
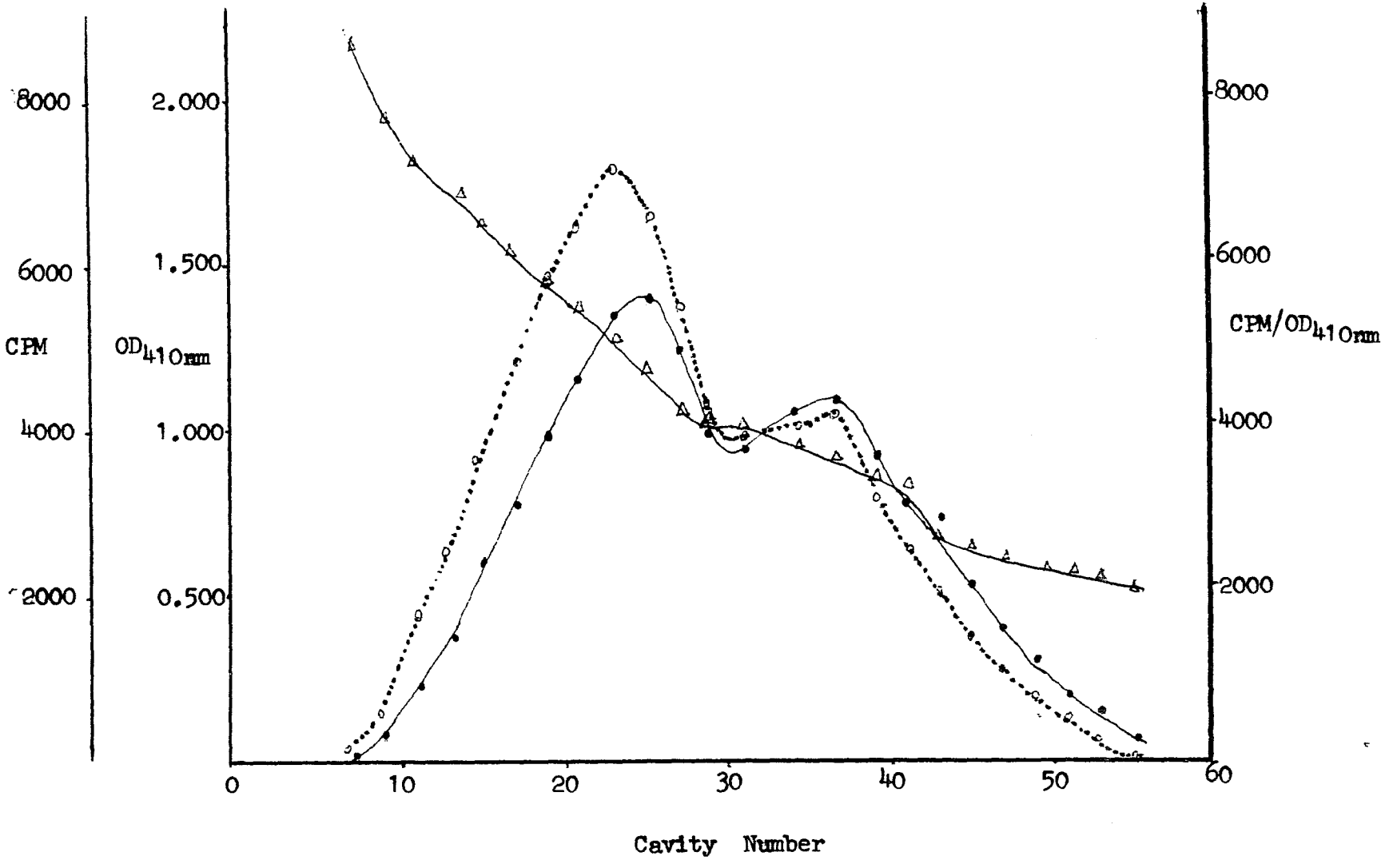

4000
3000
CPM
2000
1000
4.000
3.000
OD540nm
2.000
1.000
4000
3000
CPM/OD540nm
2000
1000
0
10
20
30
40
50
60
Cavity Number

Figure 2D. Countercurrent distribution pattern of hemoglobin-containing bone marrow cells from phenylhydrazine-injected rats; —•—•—•— = absorbance at 410 nm, -o--o--o--o- = radioactivity, —▲—▲—▲—▲— = specific radioactivity (cpm/O.D. at 410 nm). Three rats were injected with 0.5 ml phenylhydrazine (1%) on each of 4 successive days. On the 6th day serum bound ^{59}Fe was injected. 2 hours later, their bone marrow cells were subjected to countercurrent distribution (as in Figure 2A).

(Diagram is on the next page.)

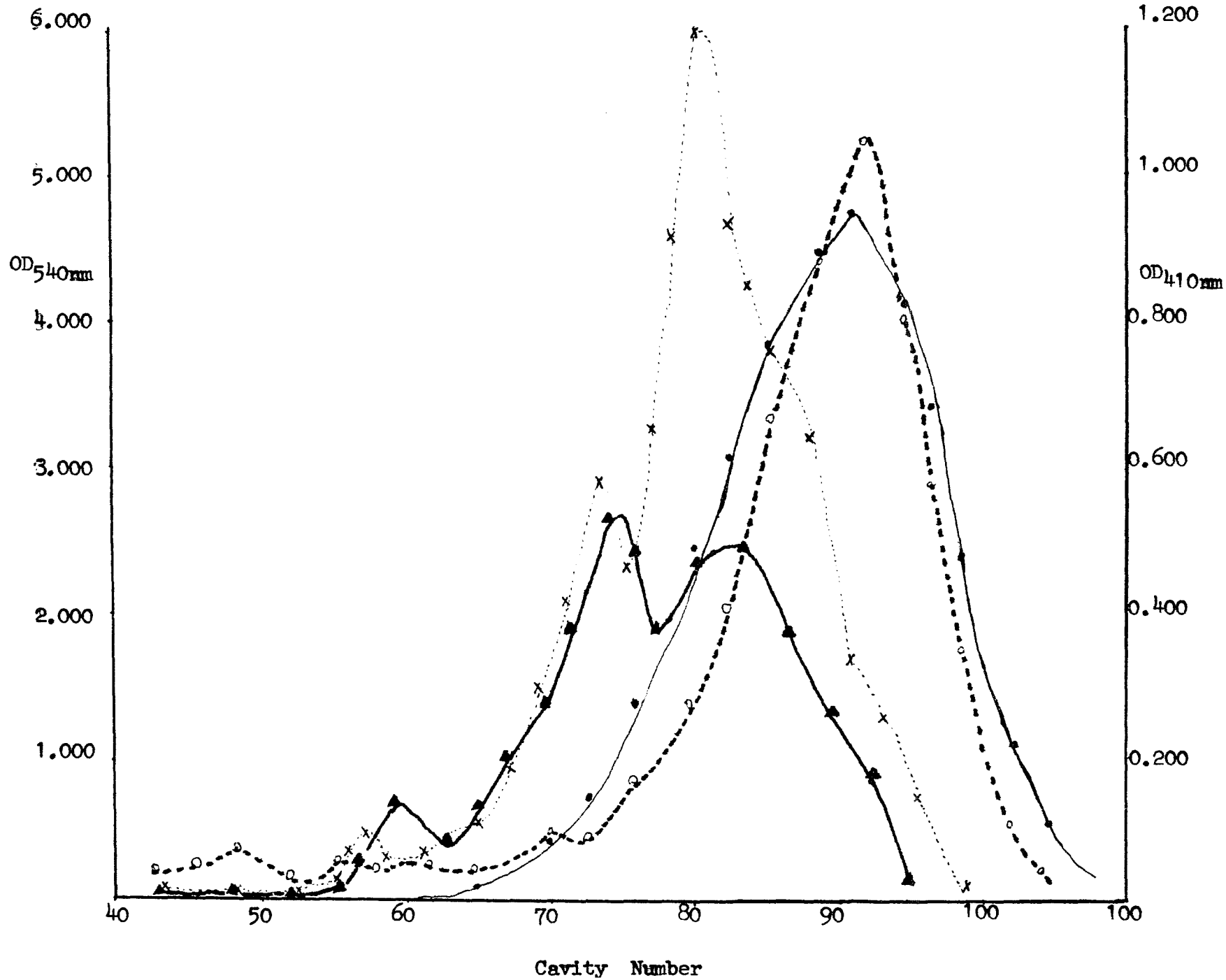

8000
6000
4000
2000
CPM
2.000
1.500
1.000
0.500
OD410nm
8000
6000
4000
2000
CPM/OD410nm
0
10
20
30
40
50
60
Cavity Number

with phenylhydrazine showed two peaks in the CCD hemoglobin pattern (Figure 2D); the smaller one is at cavity #37 and the larger one is at cavity #25. The highest radioactivity is at cavity #23. When comparing the two hemoglobin curves, of normal and phenylhydrazine-induced bone marrow cells the size of the peaks have been reversed (Figure 2B, 2D) indicating that either the number of nucleated red cell or the amount of hemoglobin was changed due to the effect of the phenylhydrazine.

Countercurrent Distribution Pattern of Bone Marrow Cells from X-Irradiated Rats

Marrow cells from splenectomized rats which were subjected to whole body X-irradiation (900 rads) three days prior to their separation contained no nucleated erythroid cells. Only one peak at cavity #92 was observed, which is the same position as that of the erythrocytes after 120 transfers have occurred in the CCD experiment (Figure 3). Walter et al. (1974) found that cells derived from the bone marrow of irradiated rats (6 or more hours after irradiation, 1000 rads) contain a large quantity of erythrocytes. Their study also indicated that irradiation at the level used (1000 rads) has no effect, measurable by Albertsson's aqueous two polymer system (i.e., partition) on the membrane surface properties of formed cells. Most readily apparent is the marked effect on cell biosynthesis itself.

Figure 3. Countercurrent distribution pattern of hemoglobin-containing bone marrow cells from X-irradiated splenectomized rats (in same phase system as in Figure 2A, 120 transfers); o--o----o--o = X-irradiated bone marrow cells, marrow removed 3 days following whole body exposure to 900 r, (absorbance at 540 nm). •——•——• = erythrocytes (absorbance at 540 nm), x--x--x--x = normal bone marrow cells (absorbance at 410 nm), ▲——▲——▲ = phenylhydrazine-stimulated rat bone marrow cells (absorbance at 410 nm). (Diagram is on the next page.)


6.000
5.000
OD_{540nm}
4.000
3.000
2.000
1.000
1.200
1.000
OD_{410nm}
0.800
0.600
0.400
0.200
40
50
60
70
80
90
100
100
Cavity Number

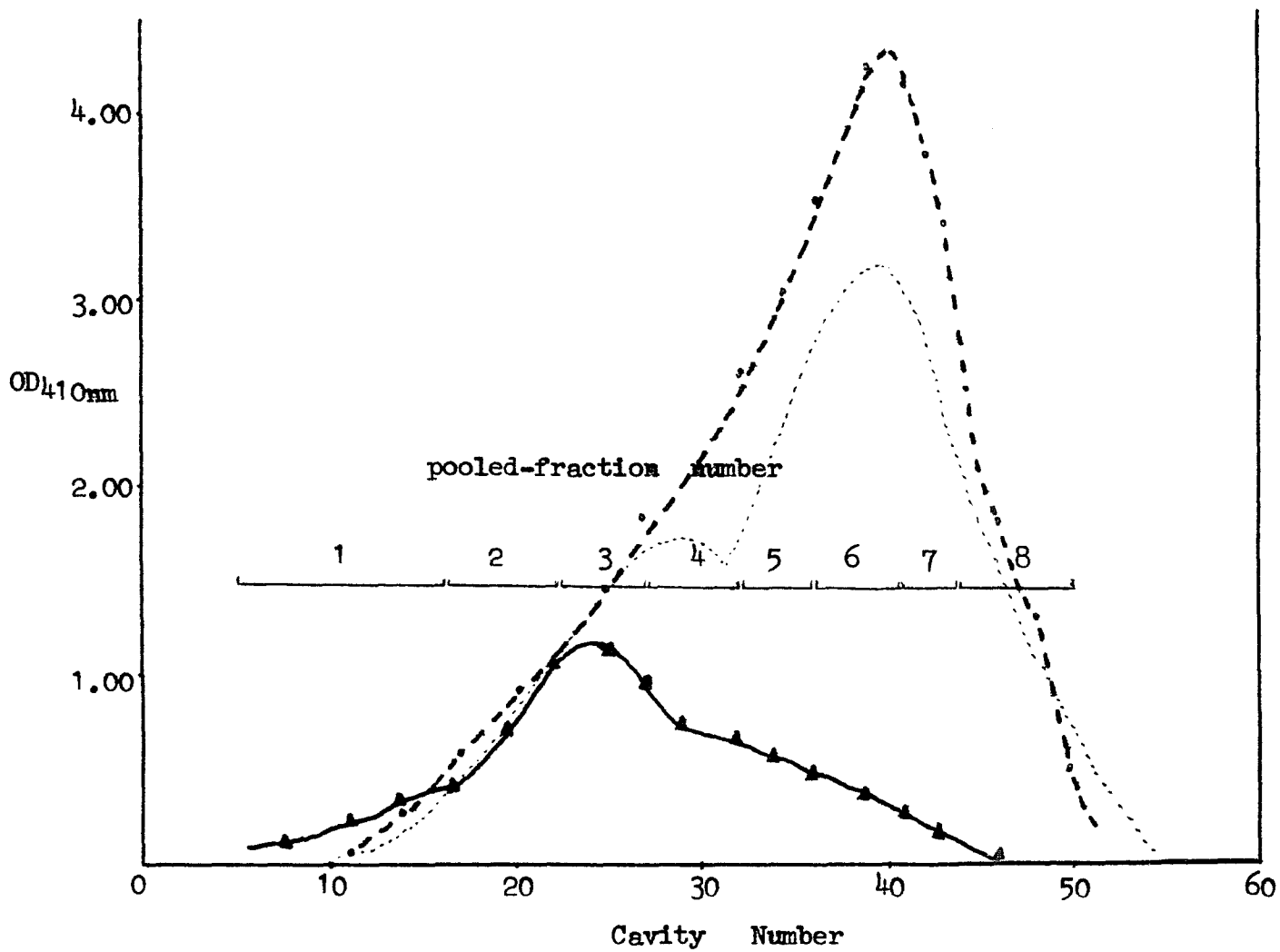
Countercurrent Distribution Patterns of Pre-Separated Bone Marrow Cells from Normal Rats

Bone marrow cells were separated in dextran T-40 (20% w/w) by density gradient centrifugation before subjection to the CCD procedure, and the optical absorption for bone marrow hemoglobin (410 nm) was measured for alternate cavities (Figure 4). The less dense bone marrow cells showed one peak at cavity #24 since the less dense bone marrow cells were very young erythroid cells. In contrast with the less dense bone marrow cells, the denser bone marrow cells showed a very high peak at cavity #40 because these cells are older bone marrow cells. The reason for this will become apparent in the next section.

DNA,RNA, Hemoglobin, and Total Protein Contents of Erythroid Cells at Different Stages of Maturation

2.0×10^9 bone marrow cells were distributed evenly into cavities #0 to 4 and #60 to 64 on a 120 transfer CCD disc. After completion of 60 transfers, the cells were collected and every 3 cavities were pooled. The pooled fractions were washed and suspended in 3 ml 0.9% NaCl (pH 7.0). 0.5 ml from each suspension was reserved for hemoglobin determination whereas 0.1 ml of each suspension was removed and diluted to 2 ml with saline (pH 7.0) for counting of cells. The remainder of the cells were divided into three parts for determination of total protein, DNA and RNA. The results are shown in Table 2, and Figure 5.

Figure 4. Countercurrent distribution patterns of pre-separated red marrow cells from normal rats; •-·-·-·-·-· = denser red marrow cells, ·-·-·-·-·-·-·-·-·-·-·-·-· = total red marrow cells, ▲—▲—▲—▲—▲ = less dense red marrow cells. Distribution of hemoglobin in denser marrow cells, total marrow cells and less dense marrow cell from normal adult rats separated by thin layer CCD, and identity of pooled fractions which were analyzed for multiple hemoglobin distribution shown in Table 3. (Diagram is on the next page.)


4.00
3.00
2.00
1.00
OD410nm
pooled-fraction number
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
Cavity Number

The average contents of total DNA, RNA, hemoglobin, and protein for each rat bone marrow cell (heterogeneous population) are 7.25×10^{-7} µg/cell, 3.36×10^{-7} µg/cell, 103×10^{-7} µg/cell, 229×10^{-7} µg/cell respectively. From cavity #4 to 9, major populations are white cells (Walter et al., 1969) mixed with aggregated cells and erythroid precursor cells, and in cavity #9 to 15 are erythroblast cells which contain considerable quantities of hemoglobin. These observations of early erythroblasts (Grasso et al., 1963) agree with the fact that the synthesis of hemoglobin commences shortly after committment (Hodgson, 1970). From cavity #15 to 24, cells have constant DNA (57.5×10^{-7} µg/cell), RNA (14.8×10^{-7} µg/cell), and protein (470×10^{-7} µg/cell) content. However, the hemoglobin content varied greatly in this region (i.e., from 20.6×10^{-7} µg/cell to 86.9×10^{-7} µg/cell). This result indicated that these cells were at the nucleated-stage and that an erythroid cell requires 50 hours to undergo several cell divisions (Tarbutt, 1969). At the same time, nucleated cells make many other proteins besides hemoglobin. At the end of this process, the cell has accumulated about one third of its final complement of hemoglobin (Matured red blood cells have approximately 280 to 300×10^{-7} µg/cell. (Schmidt, 1972; Wintrobe, 1933)). At cavity #48, the DNA content of this cavity becomes 1/60th of the DNA content of cavity #25. From cavity #25 to cavity #40, there is a gradual decrease in RNA and protein content. At cavity #40, the contents of RNA and protein have both leveled off and

this level remains constant from cavity #40 to #48. This indicated that cells at cavity #25 have lost their nucleus (and associated DNA) and therefore are not capable of RNA synthesis. The decrease in protein content results from the last cell division and the loss of many mitochondria (Gasko et al., 1965) and enzymes in the non-nucleated cells. Concomittantly hemoglobin content increased rapidly from 86.9 x 10^{-7} μg/cell to over 159 x 10^{-7} μg/cell and the concentration of hemoglobin increased rapidly from 184 to 796 μg/mg of protein. This result indicates that the residual mRNA and associated ribosomes (the protein synthesizing apparatus), after loss of the nucleus, are committed to the synthesis of hemoglobin alone. All these observations points to cavity number 25 as the point at which the nucleated cell undergoes the final cell division, loses its nucleus and enters the non-nucleated or the non-dividing cell stage. Therefore, the youngest erythroid cell has the lowest partition in this aqueous two-polymer system, and hemoglobin-containing bone marrow cell maturation is accompanied by an increase in partition of the cell in the two-phase system.

Multiple Hemoglobin Distribution in Erythroid Cells at Different Stages of Maturation

Immature erythroid cell types, differing in their degree of maturation, were obtained from the marrow of

Table 2. DNA, RNA, hemoglobin, and protein content of erythroid cells at different stages of maturation

pooled fraction	cavity number	# of cells in one cavity x 10^{+7}	µg DNA per cell x 10^{-7}	µg RNA per cell x 10^{-7}
1	5, 6, 7	0.69	63.6	16.6
2	8, 9, 10	2.29	37.9	10.0
3	11, 12, 13	3.32	37.2	9.8
4	14, 15, 16	2.21	42.5	10.3
5	17, 18, 19	1.69	57.8	14.7
6	20, 21, 22	1.04	57.5	14.8
7	23, 24, 25	1.37	57.7	13.8
8	26, 27, 28	1.46	39.2	11.5
9	29, 30, 31	1.48	27.5	7.1
10	32, 33, 34	1.67	15.9	6.0
11	35, 36, 37	2.07	7.3	4.2
12	38, 39, 40	2.44	3.6	2.6
13	41, 42, 43	2.17	2.5	1.9
14	44, 45, 46	0.71	1.3	1.8
15	47, 48, 49	0.33	0.9	1.6
unfractionated BMC		4.00	7.3	3.4

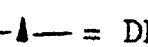
Table 2 continued on the following page.

Table 2. (continued)

pooled fraction	cavity number	µg hemoglobin per cell x 10^{-7}	µg protein per cell x 10^{-7}	µg hemoglobin / 0.1mg protein (%)
1	5,6,7	4.8	536	0.9
2	8,9,10	4.8	311	1.5
3	11,12,13	11	298	3.6
4	14,15,16	21	323	6.4
5	17,18,19	41	431	9.6
6	20,21,22	84	478	17.5
7	23,24,25	87	473	18.4
8	26,27,28	93	377	24.7
9	29,30,31	87	277	41.1
10	32,33,34	109	238	45.8
11	35,36,37	146	244	59.8
12	38,39,40	149	213	70.1
13	41,42,43	145	192	75.6
14	44,45,46	159	200	79.6
15	47,48,49	146	192	76.2
Unfractionated BMC		102	229	44.8

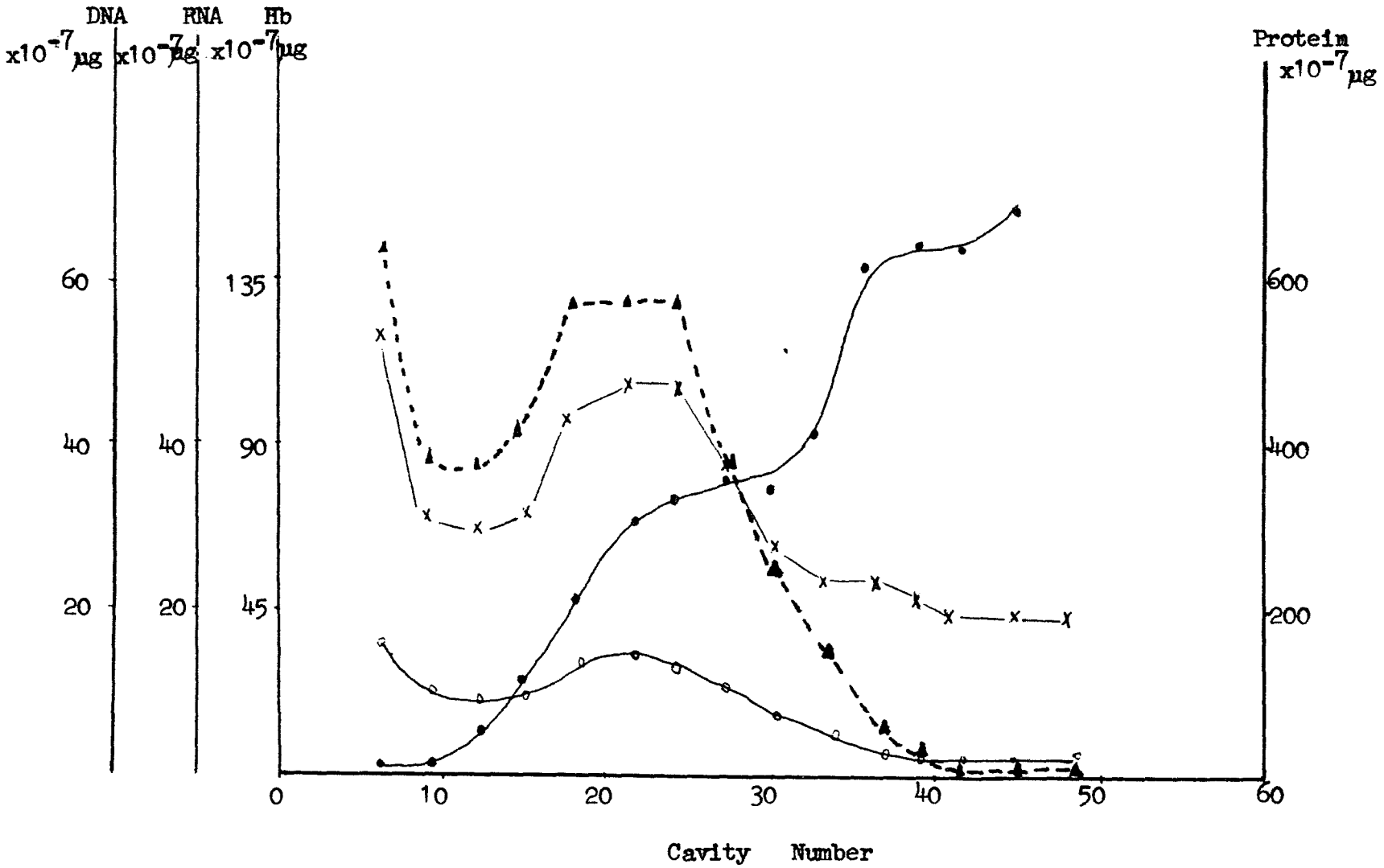
BMC = Bone Marrow Cell

Figure 5. Distribution of total protein, hemoglobin, DNA, and RNA per cell in fractions of normal rat marrow separated by thin layer CCD;

 = DNA/cell,  = RNA/cell,

 = Hb/cell, = Protein/cell.

(Diagram is on the next page.)


DNA
x10⁻⁷ µg
RNA
x10⁻⁷ µg
Hb
x10⁻⁷ µg
Protein
x10⁻⁷ µg
60
40
20
40
20
135
90
45
600
400
200
0
10
20
30
40
50
60
Cavity Number

normal adult rats. The marrow cells were fractionated according to their membrane charge to give different partition coefficients in an aqueous two-polymer phase system by countercurrent distribution. To obtain sufficient numbers of fractionated cell types for studies of the multiple hemoglobin changes during the maturation, repeated countercurrent distribution operations were employed using large scale fractions (9 ml) of less dense bone marrow cells. After one 60-transfer countercurrent distribution run, intermediate fractions were pooled as shown by the horizontal bars in Figure 4. After 18 such runs, the same pooled fractions were combined. The various hemoglobin extracts were purified by several procedures (as described in the "materials and method" section) and fractionated by isoelectric focusing (Figure 6A) from each of the pooled fractions. A quantitative determination of each of the eight fractions of marrow hemoglobins was made for pooled cells at different stages of development. The relative proportions of each hemoglobin type were determined by measurement of the areas beneath the peaks in the densitometric tracings of the non-stained gel at 540 nm (Figure 6B). The results (Figure 7 and Table 3) confirmed the visual impression (Figure 6A, B) that the proportion of Hb IV increased gradually and Hb V decreased gradually as maturation proceeded. Two acidic hemoglobin fractions (Hbs VII and VIII) decreased during erythroid cell maturation and, therefore, these fractions are associated

only with less mature erythroid cells. Pooled fraction #1 of the countercurrent distribution contained Hbs II, V, VII, and VIII and a faint band for hemoglobin Hb VI. Hbs III and IV were also found in the earliest pooled fraction but they were present in trace quantities which could be due to contamination. The second pooled fraction had all 8 hemoglobins. By comparison with components of the first pooled fraction, Hbs V, VII, and VIII of the second pooled fraction decreased whereas all the other hemoglobins increased. The Hb II content began to decrease in the third pooled fraction and that of Hb VI declined starting with the fifth pooled fraction. As shown in Figure 7, the sixth pooled fraction showed that Hb IV had the highest percentage of hemoglobin content. The last pooled fraction contained all six hemoglobin bands and only negligible amounts of Hbs VII and VIII. Hbs VII and VIII were either diluted by cell division, by the synthesis of other hemoglobins or by degradation. These data confirm the fact that Hb V biosynthesis is associated with a specific type of erythroid cell, probably a cell in the early stages of maturation. Such cells also synthesize Hbs VII and VIII which then disappear as the cells mature. The results suggest that by using Hbs V, VII, and VIII as markers, the youngest of the erythroid cell series could be isolated by this method. This conclusion is supported by the finding that the earliest fractions of marrow cells from CCD are highly

radioactive with respect to ^{59}Fe, inasmuch as Lajtha (1966) has demonstrated that the youngest erythroid cells synthesize hemoglobin most rapidly. This great advantage of having a biochemical marker (in contrast to staining and visual evaluation procedures) is obvious. Comparing the gel of the last pooled fraction with the gel of circulating red cell, hemoglobin Hb IV was observed to increase the most, indicating that Hb IV synthesis is more active than the other hemoglobins synthesized in the nearly matured cells (i.e., reticulocytes). The results also suggest that by using specific activity of hemoglobin fraction IV as a marker, the oldest hemoglobin synthesizing cells could be identified. The ratio of quantities of Hb IV to Hb V and the ratio of specific activities of Hb V to Hb IV provide a method for future studies of the metabolism of cells at a particular state of maturation.

In Vitro ^{59}Fe and ^{14}C Incorporation Experiments after Countercurrent Distribution

In order to determine whether the two polymers (dextran T-500 and polyethylene glycol) of the phase system affect cell metabolic activity, the incorporation of ^{59}Fe and ^{14}C experiments were carried out in the following manner:

Bone marrow cells were collected from 6 normal adult rats and half of these cells were placed in a test tube containing 2.5 cc of bottom phase solution and 4 cc of top

Bone Marrow Cell

Pooled-fraction number

A 1 2 3 4 5 6 7 8 B

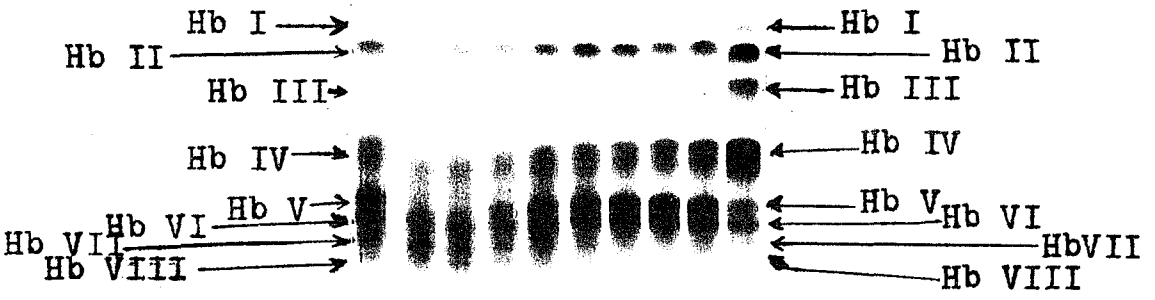


Figure 6A. Polyacrylamide isoelectric focusing gel separation of the eight hemoglobins of rat erythroid cells at different stages of maturation. The hemoglobins were extracted from cell fractions pooled as indicated by the horizontal bars in Figure 4. Approximately 0.6 mg of CO-hemoglobin from each cell type was applied to 3.5 x 140 mm acrylamide gels, pH 6 - 8. The proportions of individual hemoglobin fraction were calculated and are shown in Table 3 and Figure 7. Spectrophotometric scans of gels are shown in Figure 6B.

A = less dense bone marrow cells

B = normal red blood cells

Figure 6B. Densitometer tracing of rat hemoglobin resolved by IEF; ——— = cavity #5 - 16 (pooled fraction #1); -·-·-·- = cavity #23 - 26 (pooled fraction #3); ····· = RBC. Distribution of normal rat hemoglobins (by isoelectric focusing gel with a pH range of 6 - 8) extracted from pooled marrow cell fractions separated by thin layer CCD compared with multiple hemoglobin distribution in normal RBC. Spectrophotometer with a scanning attachment was used at 540 nm.

(Diagram on next page.)

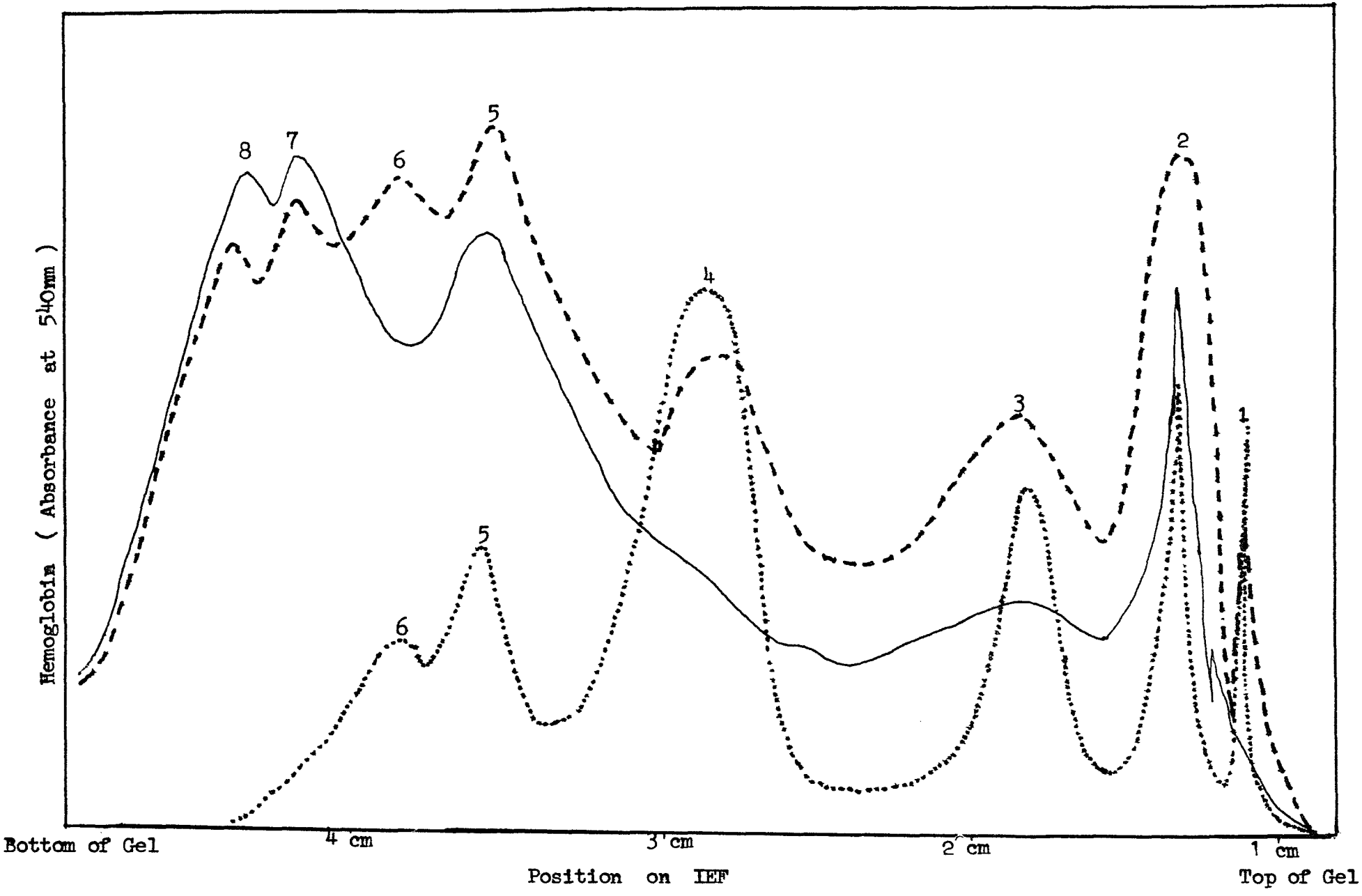

Hemoglobin (Absorbance at 540nm)
8
7
6
5
4
3
2
1
5
6
Bottom of Gel
4 cm
3 cm
2 cm
1 cm
Top of Gel
Position on IEF

Table 3. Percent distribution of the hemoglobin components of erythroid cell at different stages of maturation

pooled fractions	1	2	3	4	5	6	7	8	
pooled cavity numbers	5-16	17-22	23-26	27-32	33-36	37-40	41-43	44-52	RBC
Hbs				Percent					
Hb I	< 0.4	0.6	2.0	2.7	3.4	3.8	4.6	5.2	5.8
Hb II	12.4	19.5	16.6	14.3	12.9	12.2	13.7	14.7	16.6
Hb III	3.4	5.0	6.9	9.1	9.2	9.6	10.9	13.0	19.5
Hb IV	3.8	7.9	13.2	16.7	20.8	24.8	27.3	28.4	40.1
Hb V	31.9	24.1	21.3	20.4	20.9	21.1	19.1	17.6	11.1
Hb VI	5.3	9.6	15.4	19.4	19.9	18.9	18.2	16.6	6.7
Hb VII	23.1	18.4	14.1	10.2	8.4	5.8	4.0	3.1	<0.4
Hb VIII	20.1	14.7	10.8	7.2	4.6	3.8	2.2	1.7	<0.4

Normal rat bone marrow cells had been separated by thin layer CCD. Hemoglobins were separated by IEF. The values represent percentage distribution calculated from peak areas of densitometric tracings of analytical polyacrylamide gels. Results are based on data of at least three IEF experiments.

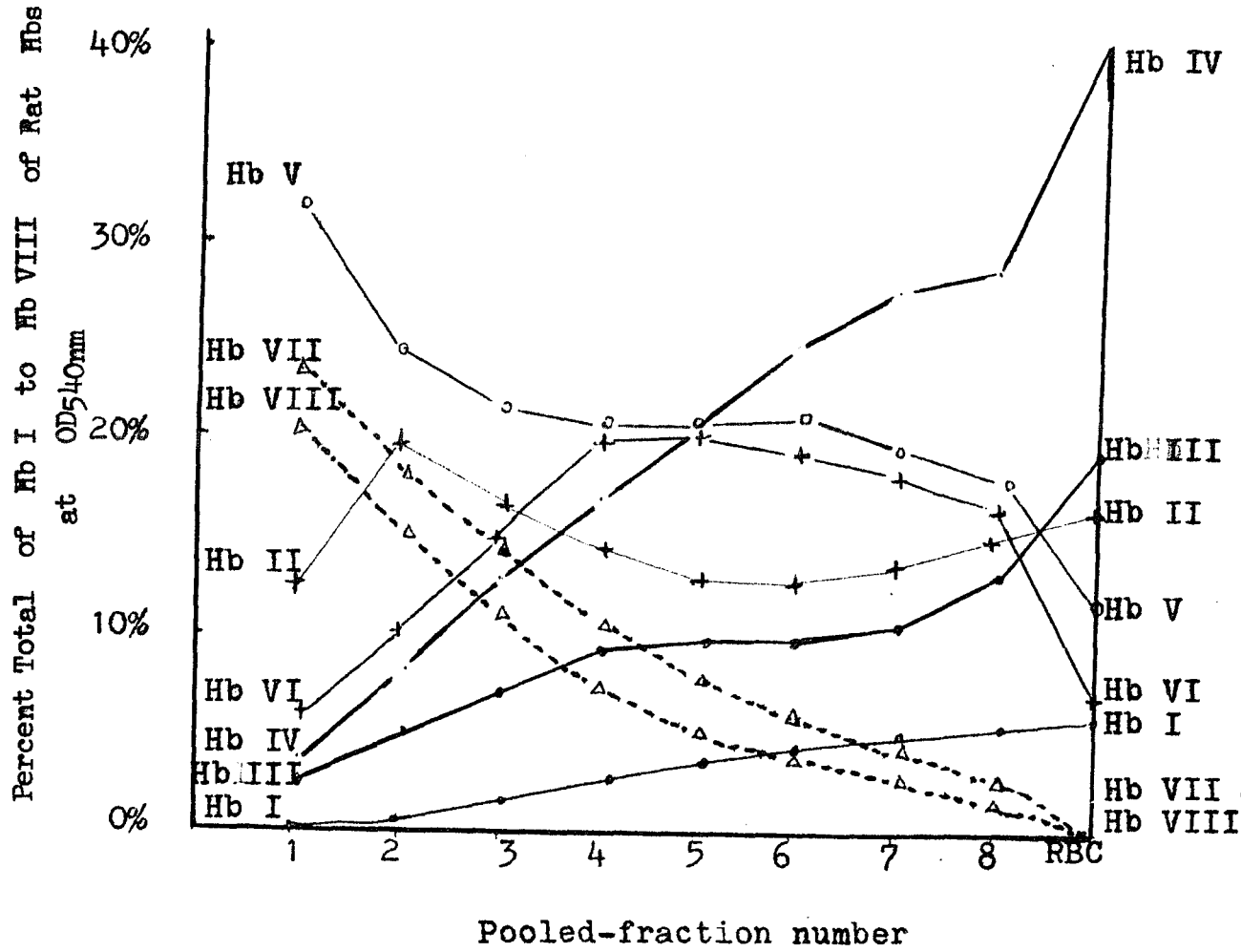


Figure 7. Percent total of Hb I to Hb VIII of rat erythroid cells at different stages of maturation
Normal rat bone marrow cells were separated by thin layer CCD. Hemoglobins were isolated from the pooled-fractions and separated by IEF. The values represent percentage distribution calculated from peak areas of densitometric tracings of analytical IEF gel. The values of percentage are the same as that of Table 3.

phase solution. The test tube was sealed with parafilm and placed on the top of the CCD shaking table and the contents were shaken for 45 seconds. Afterward, 6 minutes were allowed for the cells to settle. This procedure was repeated 60 times in the cold room (without cell transfer) to simulate the conditions of the CCD experiment, so that these cells would be exposed to the same conditions as those in the CCD experiment. The other half of the bone marrow cells was suspended in two-phase system buffer solution (0.09 M phosphate, 0.03 M NaCl, pH 6.8) and was placed in the cold room to serve as control. The following morning, both the experimental and control cells were washed three times with saline. The control and experimental cells were incubated in ^{59}Fe medium (6×10^6 cpm/medium) for various lengths of time (Figure 8). The medium was prepared according to Borsook et al. (1959). After cells were washed three times, crystallized hemoglobin was isolated and dissolved in Drabkin solution, determinations of hemoglobin and radioactivity were then carried out. The results showed that the experimental cells and control cells had almost identical activities (see Figure 8) which suggests that the two polymers (dextran and polyethylene glycol) and the mechanical motion of countercurrent distribution did not impair their biological activities. In other words, cells mixed with the two polymers, shaken, and then transferred in the CCD instrument for half a day possessed the same metabolic ability (at least for

hemoglobin synthesis) as those cells which were immersed in the phosphate buffer for the same period of time. The advantage of the CCD method is that this method minimizes trauma to the cell due to the physiological inertness of the two polymers which themselves do not change the isotonicity of the buffer greatly. Also the liquid-liquid interface does not produce any denaturation, as many other interfaces do, presumably because of its extremely low interfacial tension (0.1 to 1 x 10^{-4} dyne cm^{-1}; Albertsson, 1971). When the metabolic activities of the CCD experimental bone marrow cells were compared with those of freshly prepared bone marrow cells, the specific activity of experimental cells reached 80% to 90% of the specific activity of freshly prepared bone marrow cells when the CCD experimental bone marrow cells were pre-incubated for a fairly long period of time (i.e., 80 min). As also shown in Figure 9, the freshly prepared bone marrow cells showed little decrease in specific activity whereas the experimental bone marrow cells showed increasing specific activity with increasing pre-incubation time, probably because the experimental cells coated with the dextran-polyethylene glycol mixture need to be "stripped" of these compounds prior to allowing them to incorporate compounds from the medium. These results indicate that the experimental cells may be affected by the medium and the cold, and either effects can be reversed with incubation. These comparisons also show that the younger erythroid cells tend to be more

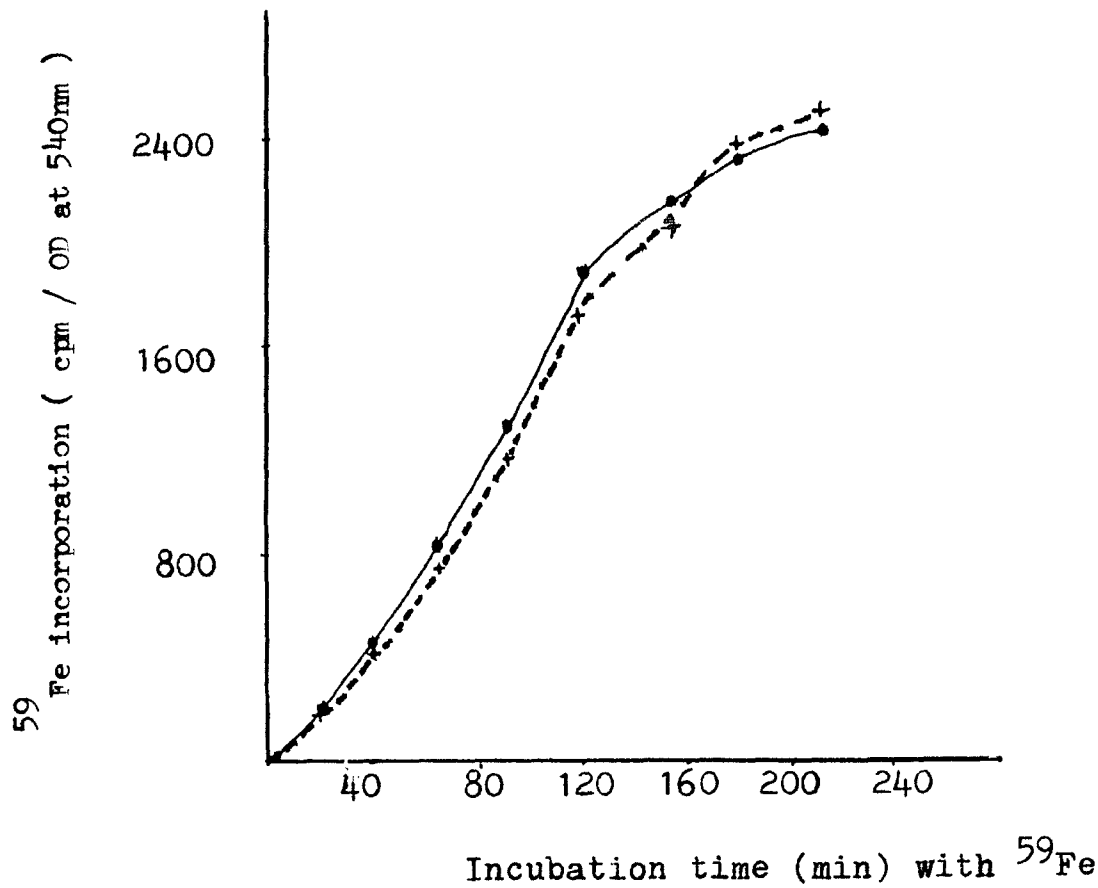


Figure 8. Comparison of ^{59}Fe uptake by normal rat marrow cells treated by the CCD procedure and control untreated cells; +---+---+--- = control (no CCD) marrow cells; •———•———• = experimental (CCD) marrow cells. Both control and experimental marrow cells were isolated from rats a day before incubation. Control cells were suspended in phosphate buffer overnight in the cold room. Experimental cells remained in the CCD 2-polymer phase system overnight before radioactive iron incorporation.

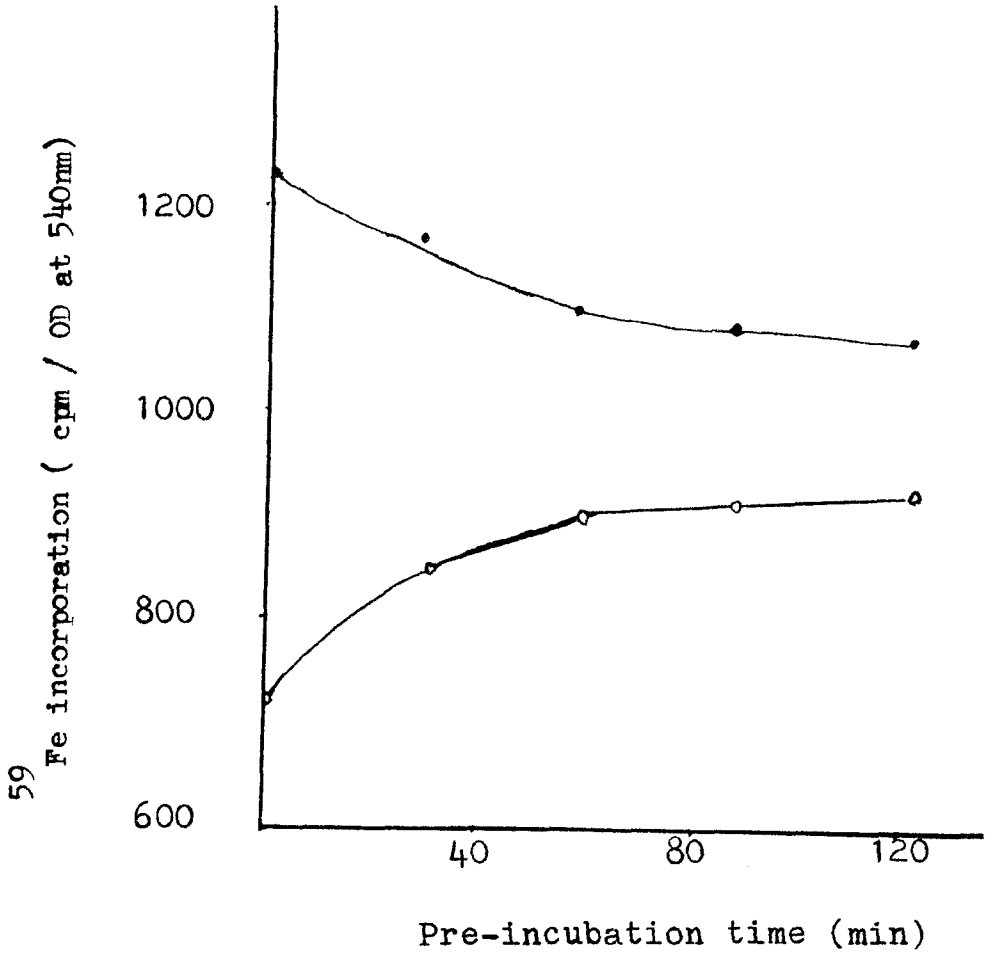


Figure 9. Comparison of <u>in vitro</u> ^{59}Fe incorporation into hemoglobin of freshly prepared bone marrow cells which have been subjected to the CCD procedure; •——•——• = freshly prepared bone marrow cells isolated from rats and incubated immediately; o——o——o = CCD experimental bone marrow cells subjected to CCD procedure for 7 hours a day before incubation. Both freshly prepared bone marrow cells and CCD experimental bone marrow cells were incubated with non-radioactive medium for various lengths of pre-incubation time followed by a one hour incubation in ^{59}Fe medium.

fragile and thus, the freshly prepared bone marrow cells were lysed after a long period of pre-incubation. The lysis of the fragile young erythroid cells in the solution is a phenomenon which often occurs and cannot be avoided even if these cells were kept in the incubation medium.

The bone marrow cells were collected from the CCD run and every third cavity was collected and pooled. The pooled fractions were each incubated in the ^{14}C-leucine medium. After incubation, the cells were washed 5 times with saline and then lysed. Protein was dissolved into 0.1 N NaOH and then precipitated with 10% TCA. The protein precipitate was then washed successively with (1) 10% TCA at 80 - 90°C for 30 minutes (2) acetone (0.5% HCl) at -20°C (3) ether ethanol (1:1) at 35°C for 30 minutes and (4) ether for 15 minutes. The precipitate was then air dried and dissolved in saline. The concentration of total protein of cells was measured by the Lowry procedure (Lowry et al., 1951). Radioactive content (^{14}C-protein) was measured in a Beckman LS-150 liquid scintillation counter. Figure 10, curve (A), represents experiments carried out for an hour incubation in non-labelled leucine medium after which the cells were transferred to ^{14}C-leucine medium for another hour. Curve (B) represents experiments done in half hour of pre-incubation in non-labelled medium where upon the cells were transferred to ^{14}C-leucine medium for a half

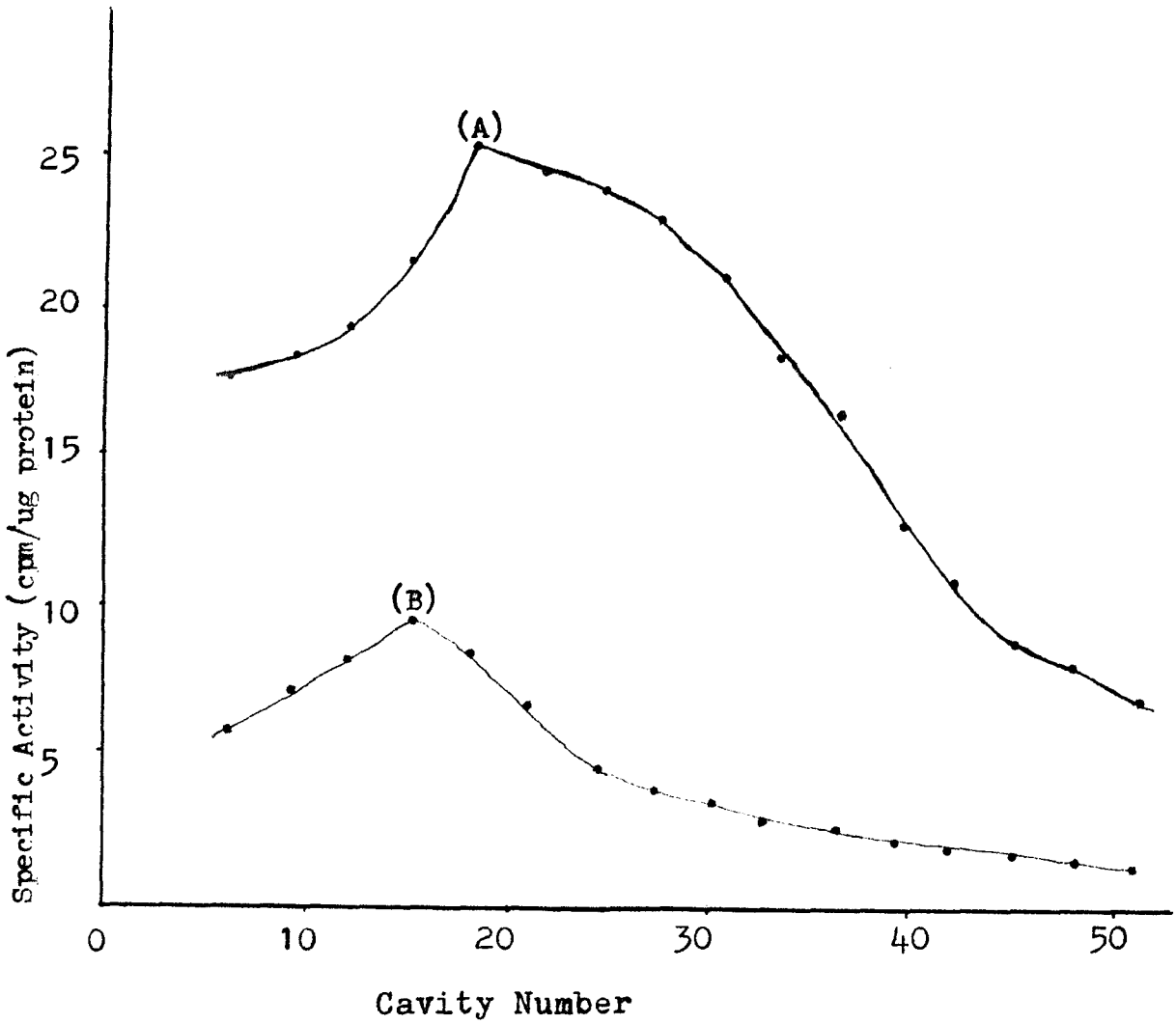


Figure 10. *In vitro*, ^{14}C-leucine incorporation into the total protein of marrow cells which were fractionated by CCD; curve A = experiments carried out for a one hours incubation in non-labelled leucine medium after which the cells were transferred to ^{14}C-leucine medium for another one hour incubation; curve B = experiments done in a half hour of pre-incubation in non-labelled medium whereupon the cells were transferred to ^{14}C-leucine medium for another half hour incubation.

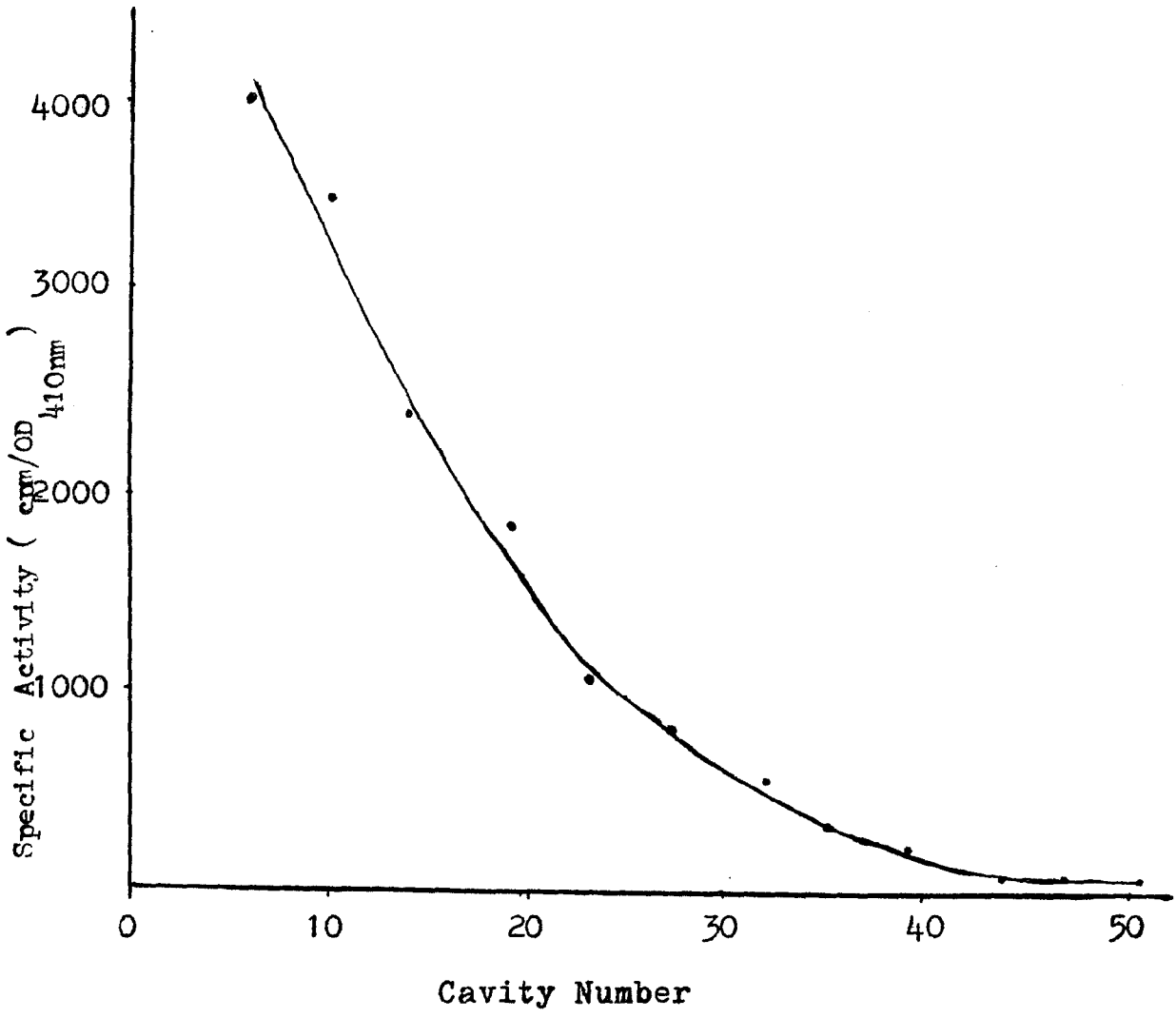


Figure 11. In vitro, ^{59}Fe incorporation into hemoglobin of fractionated marrow cells by CCD. Bone marrow cells were separated by CCD as in Figure 2B. Fractionated cells were pre-incubated for one hour with non-iron medium and then transferred to ^{59}Fe medium for another hour. Bone marrow hemoglobin isolated from CCD fractions was measured for its specific activity (cpm/O.D. 410 nm)

hour incubation. Figure 11 showed cells from CCD experiments which were incubated with ^{59}Fe (one hour pre-incubation followed by one hour incubation in ^{59}Fe medium). In this case, ^{59}Fe was incorporated into the bone marrow hemoglobin. The bone marrow cells were lysed in ice cold water and hemoglobin crystal was then isolated and dissolved into Drabkin solution. The specific activities of the hemoglobin were measured.

In vitro, both ^{14}C incorporation into total protein (Figure 10) and ^{59}Fe incorporation into hemoglobin (Figure 11) showed that the higher specific activity was found on the left side of the extraction "train". This result is consistent with the in vivo labelling experiment shown in Figure 2B. All these data indicate that the left side of the extraction "train" contains younger erythroid cells than the erythroid cells on the right side. In ^{14}C-leucine incubation experiments (Figure 10), specific activity of ^{14}C-protein decreased from cavity #15 to #5 because white cells appeared in that region. These data indicated that the cells still had good metabolic activity even after they had been subjected to countercurrent distribution. Thus, in general, it is possible to perform CCD of cells in these polymer two-phase systems without apparent deleterious effects on metabolic integrity.

^{59}Fe and ^{14}C-Leucine Incorporation into Rat Hemoglobin In-Vitro

Both the spleen and the bone marrow are erythropoietic organs in adult rats (Mazur, 1968, 1969) and have approximately the same distribution of hemoglobin components (Stein et al., 1971). Spleen is easily studied since it can be obtained in relatively large quantities. In the present study the incoproration of isotopic precursors into the six (or eight) hemoglobins of bone marrow cell suspensions were investigated. Bone marrow cells were isolated from heparin-injected rats, washed thoroughly in cold 0.9% NaCl and incubated at 37°C in a medium prepared according to Borsook et al. (1957) containing the isotopic precursor. After 90 min the marrow cells were washed free of excess isotope and the hemoglobin extracted, purified and separated into its multiple components by preparative isoelectric focusing (IEF). Each fraction was eluted and its specific activity determined as counts per min per mg of hemgolboin. The specific activities of the six hemoglobin fractions appeared to be identical except for Hbs VII and VIII in all instances, both when the cells were incubated with serum-bound ^{59}Fe, which was incorporated into heme of hemoglobin, or incubated with ^{14}C-leucine, which was incorporated into the globin moiety. The specific activities are shown in Taole 4. Each of the hemoglobins except for Hbs VII and VIII is synthesized at a rate proportional

to its relative distribution and that the precursor pool for iron and leucine is the same in all erythroid cells except for those of Hbs VII and VIII. From the above results it can be concluded that all different types of hemoglobins are synthesized to the same specific activity since the total spleen and total marrow contain all stages of erythroid cells undergoing cell differentiation and maturation. On the other hand, both Hbs VII and VIII have higher specific activities than the other types of hemoglobin specific activities. These results suggest that very young cells which synthesize Hbs VII and VIII are more permeable to ^{59}Fe and leucine, or alternatively, they may be the precursors of the other hemoglobins.

The following experiments deal with the reticulocyte:

Within 2 or 3 days, reticulocytes mature into erythrocytes accompanied by a complete cessation of RNA synthesis, a loss of the mitochondrion (Gasko et al., 1972), and a progressive degradation of the polyribosomes and at least one of the initiation factors required for protein synthesis (Herzberg et al., 1969). The reticulum from which this stage derives its name is a remnant of the protein-synthesizing system and disappears within a day or two after the reticulocyte goes into the circulation. Rat red cells relatively rich in reticulocytes, were obtained from anemic animals (above 200 gms) that had been given subcutaneous injections of 0.5 ml of a 1% solution of phenylhydrazine-HCl on each of 4 consecutive days.

Blood was collected in isotonic heparin solution 5 days after the last injection. Under these conditions, the red blood cells contained a relatively large number of reticulocytes (approx. 55% to 45% of total red cells) compared to the normal rat peripheral red blood cells, since reticulocytes from phenylhydrazine-injected rats mature into erythrocytes very rapidly (Walter et al., 1972a). 1 ml of washed, packed reticulocyte-rich red cells was incubated at 37°C for 2 hours in a medium containing ^{14}C-leucine (20 μCi, specific activity = 315 mCi/mM). Hemoglobin was extracted and crystallized followed by a purification using G-75 and DEAE-cellulose chromatography. Multiple forms of hemoglobin were separated by IEF and specific activities of each fraction were measured (See Table 4). Ratios of specific activities of each of rat hemoglobin fractions I, II, III, V, and VI to the specific activity of hemoglobin fraction IV were calculated.

According to Stein's data (1971) for the amino acid composition of the six rat hemoglobin components, all six different hemoglobins have almost the same number (68 to 70) of leucine residues in each hemoglobin. For this reason, calculations of specific activity would not be affected by the use of total hemoglobin of each type instead of the total amount of leucine. It is likely that the reticulocyte-rich red cells also contain a certain amount of nucleated erythroid cells capable of synthesizing

Table 4. *In vitro* incorporation of ^{59}Fe and ^{14}C-leucine into hemoglobin of rat bone marrow cells and reticulocyte-rich RBC

	Bone Marrow Cells Specific Activity		Reticulocyte-rich RBC	
Hb	$\frac{^{59}Fe\ cpm}{mg\ Hb} \times 10^3$	$\frac{^{14}C\ cpm}{mg\ Hb} \times 10^4$	specific activity $\frac{^{14}C\ cpm}{mg\ Hb} \times 10^4$	ratio of Hb x/Hb IV
I	1.57	1.34	1.00	0.89
II	1.53	1.33	0.79	0.69
III	1.59	1.35	0.98	0.86
IV	1.52	1.32	1.14	1.00
V	1.62	1.36	0.64	0.56
VI	1.67	1.45	0.82	0.71
VII	1.96	1.87	—	—
VIII	2.23	2.07	—	—

hemoglobin. Thus the data as described in Table 4 will be subjected to some error. Despite this, the results demonstrated different specific activities for six rat hemoglobins. These were found to vary in the following order relative to Hb IV, which had the highest specific radioactivity:

Hb IV — 1.00
Hb I — 0.89
Hb III — 0.86
Hb VI — 0.71
Hb II — 0.69
Hb V — 0.56

Due to the heterogeneity of the red cell populations and the effect of phenylhydrazine, it is difficult to distinguish which hemoglobin has the higher activity when comparing Hbs I and III or Hbs VI and II but Hb V can be distinguished definitely from that of Hb IV.

In Vivo Isotope Incorporation into Rat Hemoglobin

Synthesis of the multiple hemoglobins was followed in vivo by means of radioactive iron labelling of bone marrow erythroid cells. Hemoglobin was prepared from blood samples drawn from splenectomized rats on successive days, after an intravenous injection of serum-bound ^{59}Fe. According to Stein's (1971) data, the specific activity of Hb IV was much higher than that of Hb V in circulating red blood cells soon after ^{59}Fe administration, and as the number of days increased after ^{59}Fe injection, the

specific activity of Hb V slowly approached that of Hb IV. By day 10, the specific activity of Hb V equalled the specific activity of Hb IV. These results can be interpreted to indicate that the reticulocytes were synthesizing Hb IV at a higher rate relative to Hb V. The same experiments were carried out and similar observations were made (Table 5).

In Vivo, ^{59}Fe Incorporation into Hemoglobin during Suppression of Endogenous Production of Erythropoietin

Rats made polycythemic by infusion of erythrocytes and starved rats had a marked depression of hemoglobin synthesis activity in their erythroid cells. Female CFN - strain rats (230 - 250 gms) were made polycythemic by intraperitoneal injections of 10 ml of packed, washed red cells from donor rats of the same strain, on the 1st, 2nd, and 5th day (a total of 30 mls). Hemoglobin concentrations were measured before each injection and also on the 7th day by the standardized hemoglobin cyanide method (O.W. Van Assendelft, 1972). The concentration (gm/100 ml) of hemoglobin were 12.8 ± 0.5, 16.7 ± 1.9, 18.7 ± 0.8, and 19.3 ± 0.9 for the 1st, 2nd, 5th, and 7th day, respectively.

After measurement of hemoglobin on the seventh day (re-marked as day 0), the rats were given ^{59}Fe-serum (6×10^6 cpm). At 7 hrs after injection, labelled hemoglobin was not detected in the blood stream of the polycythemic rats. On day 1, the rats showed 1/20th the

capability of the control rats' in incorporation of ^{59}Fe and from day 4 to day 14, one tenth the capability of normal rats' was observed. Rats which were starved for 5 days had the same poor ability to incorporate ^{59}Fe. Quantitiative insufficiency of hemoglobin may be the result of a long period of protein starvation and decreased globin synthesis. The specific radioactivities of Hbs IV and V were measured and the ratios were calculated as shown in Table 5. The data (Table 5) showed a depressed erythropoietic activity which means that few young red cells were released into the peripheral circulation, and only a small amount of hemoglobin was synthesized. Because of these two factors, the ratio (V/IV) of specific activities was below 80% as long as day 14. When rats were given intraperitoneal injections of 10 ml packed red cells after ^{59}Fe administration, at 2 hrs and on day 1 and day 4, the results were similar to that of the control normal rats for the first 4 days, but after that time the ratio (Hb V/Hb IV) of specific activity remained at the same level for a long period of time (at day 18, the ratio was still at 0.85). When rats were starved after ^{59}Fe injections, the results were found to be similar to that of the results for the control rats. This is probably because a short period of starvation does not completely stop hemoglobin synthesis in erythroid cells as compared to that seen in polycythemic rats. Also, malnutrition may cause hemolysis in the red cell inducing the "young"

erythroid cells to be released from the bone marrow.

Table 5. *In vivo* incorporation of ^{59}Fe into hemoglobins of normal, polycythemic, and starved rats.

Time	Specific Radioactivity Ratio (Hb V/Hb IV)			
	Control	A	B	C
7 hrs	0.59 ± 0.02	0/0	0/0	0.51 ± 0.02
day 1	0.81 ± 0.00	0.35 ± 0.03	0.45 ± 0.19	0.79 ± 0.03
day 4	0.85 ± 0.01	0.65 ± 0.07	0.70 ± 0.08	0.84 ± 0.02
day 7	0.92 ± 0.04	0.65 ± 0.05	0.78 ± 0.01	0.78 ± 0.02
day 11	0.97 ± 0.01	0.73 ± 0.04	——	0.79 ± 0.04
day 14	1.02 ± 0.03	0.77 ± 0.05	——	0.84 ± 0.05
day 18	——	——	——	0.85 ± 0.06

Ratio of specific activity of rat Hb V to the specific activity of Hb IV, with time, after *in vivo* labelling of marrow erythroid cells with serum-bound ^{59}Fe. These values are averages from four control rats with average deviations reported. All other values are the averages from two rats which have had the same treatment.
A = pre-treated rats to produce polycythemia; B = pre-treated rats to induce starvation; C = rats post-treated to produce polycythemia.

DISCUSSION

Erythropoiesis in Normal Rats

Rifkind et al. (1969) has shown the cytological and ultrastructural changes in the erythroblast which characterize the differentiation of hepatic erythropoiesis, and Borsook (1969) has reviewed the sequence of events that occur during erythropoiesis, including the stages during which hemoglobin synthesis proceeds. Goldwasser (1975) proposed a model of hemopoietic stem cell differentiation and indicated that the origin of the red cell is a "pluripotential" stem cell in the marrow which is triggered to become the "committed" stem cell by unknown stimuli. These committed cells would then represent the self-generating precursors of the granulocyte committed cells, thrombocyte committed cells and erythrocyte committed cells. The erythrocyte committed cells are represented by proerythroblasts, which are characterized by their capacity to respond to erythropoietin stimulation with an increase of cell proliferation (Paul and Hunter, 1968) and with the synthesis of globin messenger RNA (Chui et al., 1971). Hemoglobin or globin cannot be synthesized and is not detected in this largest and earliest recognizable developing erythroid cell —— the proerythroblast (Terada et al., 1972). This stage is followed by the basophilic erythroblast, the polychromatic erythroblast, the orthochromatic normoblast, reticulocyte and the erythrocytes. Hemoglobin synthesis probably begins

in the cytoplasm of the immediate progeny of the proerythroblast, the basophilic erythroblast, but the first stainable trace of hemoglobin marks the transition to the polychromatophilic stage. The orthochromatic erythroblast is the last nucleated stage. After this point, cell division has ceased and RNA synthesis is minimal, while protein production declines in the non-nucleated reticulocyte and is absent entirely in the erythrocyte. The reticulocyte which is incapable of expressing other genes except for globin chains appears in the circulation in small numbers in the normal animal and is the immediate precursor of the mature red cell (erythrocyte). Based on a quantitative analysis of cells which were fractionated by CCD (figure 4 and 5), the "young" nucleated cells are separated gradually from the "old" non-nucleated cells of the erythroid series. Maturation, which includes loss of the nucleus and an increase in the quantity of hemoglobin per cell, apparently is accompanied by an increase in cell membrane charge and, therefore, of partition.

Hemoglobin Biosynthesis in Normal Rats, Starved Rats and Polycythemic Rats

In Table 5, hemoglobin was prepared from blood drawn from splenectomized rats on successive days after an intravenous injection of serum ^{59}Fe. In vivo incorporation of radioactive iron into hemoglobin components

was measured by the specific radioactivity of each hemoglobin at various time intervals. At the earliest period following injection of serum-bound ^{59}Fe, the specific radioactivity of Hb IV in RBC was the highest, whereas that of Hb V was the lowest. Specific radioactivity of the remaining fractions varied between those of Hbs IV and V.

The results of the in vivo labelling experiments can be interpreted readily on the assumption the Hb V is synthesized most actively in the "youngest" erythroid cells which reside in the marrow while undergoing differentiation for the longest period of time prior to their entrance into the circulation, whereas Hb IV is most actively synthesized in older erythroid cells which enter the marrow after only a short period of time.

Jacobson et al. (1959) had shown that red cell production is regulated by the plasma level of erythropoietin and that production of this hormone is regulated by the balance between tissue oxygen supply and demand. Thus the oxygen consumption and oxygen demand appear to determine the level of erythropoiesis. Naets (1963) has demonstrated that rats starved for 3 days sustain a decrease in metabolic rate along with a decrease in oxygen comsumption. Naets (1963) has also demonstrated that reduced erythropoietin response was observed in starved and polycythemic rats due to hypoxia. In this

study, we found that the transfusion-induced polycythemic rats and rats starved for 5 days have only approximately 1/20th the capability of the control rats to incorporate ^{59}Fe into RBC 24 hrs after ^{59}Fe injection. A decrease in erythropoiesis of starved rats is due to the need for less oxygen by these animals. Decreased erythropoiesis in the hypertransfused polycythemic rat is due to the animal's increased oxygen carrying capacity in relation to its need. Table 5 indicates that polycythemic rats and starved rats have lower values of specific radioactivity ratios (Hb V/Hb IV) than that of control animals. Because erythropoiesis is suppressed by the procedure of hyper-transfusion and starvation, only a small amount of hemoglobin synthesizing cells are released into the circulation and these cells would be the relatively mature cells which had synthesized Hb IV most actively. The results, once again, confirm the non-uniform bio-synthesis of multiple rat hemoglobins.

Normal Rat Erythroid Cell Maturation and Aging Patterns as Separated by Thin Layer CCD

Figure 2A, the combination of radioisotopic techniques and thin layer CCD, indicated that the CCD curve of labelled "young" reticulocytes (possessed the highest specific activity) are displaced to the left of the erythrocyte distribution when first released into blood. The specific radioactivity was decreased from cavity #15 to #30 as the

reticulocytes age over a short period of time (i.e., 24 hrs or so). Their distribution curves move futher and further to the right (i.e., to #56). The oldest reticulocytes are displaced to the right of the erythrocyte distribution curve and have the highest partition coefficient of any RBC in the circulating blood. The reticulocyte then becomes a mature, young erythrocyte and the process is reversed with the partition coefficient of erythrocytes diminishing with age. As shown in Figure 2A, specific activity goes down from extraction cavity #56 to #30. The oldest erythrocyte has a partition coefficient very close to that of the youngest reticulocyte around cavity #25 to #30. Walter and Selby (1966) have done the detailed RBC separation experiments on CCD and found the same subtle alterations in the rat red blood cell membrane during maturation and aging.

In Figure 2B, two sub-populations of hemoglobin-containing bone marrow cells were separated: the left (cavity #11 to #31) containing both nucleated cells and non-nucleated cells and a larger peak to the right (cavity #31 to #50) containing only non-nucleated cells. Therefore, we can conclude that the cells under the left peak are precursors of the cells under the right peak. This can be proved by the specific radioactivity curve in Figure 2B, density properties of cells in Figure 4 and a decrease in DNA content and an increase in the quantity of hemoglobin per cell in Table 2 and Figure 5.

Walter et al. (1973) also had found the same age distribution by counting nucleated cells microscopically for each cavity. There are two or three different hemoglobin-containing nucleated cells present in the bone marrow. These nucleated cells have not yet been distinguished on slides from the separated bone marrow cells by thin layer CCD. The cells under the right peak are released into the circulation. This is evidenced by the fact that the youngest reticulocytes in the peripheral blood have an identical position on CCD at cavity #35 to #40 (compare positions of right peak in Figure 2B to red cell distribution in Figure 2A). Thus, the partition of cells is primarily determined by the cell's surface charge. Maturation of bone marrow cells to the youngest reticulocytes, followed by the oldest reticulocytes is accompanied by an increase in surface charge. Aging of erythrocytes, on the other hand is attended by a decrease in surface charge.

Rat Erythroid Cell Maturation Pattern in Phenylhydrazine-Injected Rats as Separated by Thin Layer CCD

Animals repeatedly injected with phenyhydrazine become severely anemic and reticulocytes may account for more than 95% of the peripheral RBC population. Such cells are of excessive size, a possible consequence of skipped divisions during erythroid cell proliferation in the bone marrow. The specific radioactivity (^{59}Fe) of

phenylhydrazine-induced bone marrow cells and peripheral red cells are greater then those of the corresponding normal marrow cells due to the presence of more nucleated red cells in the marrow and more abnormal reticulocytes in the circulation of such stressed rats (Figure 2C, D). The evidence for the higher specific activities in stressed rats is that they have more nucleated erythroid cells (Figure 2D) and their bone marrow cells are extruded as abnormal reticulocytes in the circulation, as shown in the CCD curve of Figure 2C. In Figure 2C, some cells (in cavities #15 to #31) have partition properties and membrane properties similar to nucleated and non-nucleated bone marrow cells. It can be concluded that the phenylhydrazine stress causes the bone marrow to release cells into the circulation from both the left peak and the right peak (Figure 2B, D). Also, the cells from cavities #15 to #35 in Figure 2C correspond to the left peak of then normal bone marrow cells of Figure 2B which contain the highly reticulated (young) reticulocytes. These reticulocytes are most active in protein synthesis as shown by high specific radioactivities. The cells to the right contain reticulocytes which contain much less reticulum as compared to the cells of the left side. These data indicate that the cells at the left side are "younger" than the cells at the right side.

Non-Uniform Biosynthesis of Multiple Hemoglobins in Adult Developing Erythroid Cells

The differences in distribution of hemoglobins (by IEF) from different fractions of bone marrow cells which were separated by CCD (Table 3) suggests a non-uniform biosynthesis of the multiple hemoglobins during erythroid cell development. Figure 6B represents the relative distribution of the multiple hemoglobins by optical density tracing of each gel. The earliest ("youngest" erythroid cells) fractions (cavity #7 - 16) contained essentially only Hbs V, VII, and VIII. The latter two hemoglobins are confined to the marrow and are not present in detectable amounts in the circulating red cells. The relative decrease of Hbs V, VII and VIII along with the increased appearance of other hemoglobins are evident in the intermediate fractions. The latest fractions ("oldest" erythroid cells) have a distribution of hemoglobins approaching that seen in adult red cells.*

[*The phenomenon of non-uniform biosynthesis of multiple hemoglobins is not confined to the rat. This is confirmed by similar experiments done with the guinea pig. In our laboratory, Win Lin has separated marrow erythroid cells into "young" and "old" cells by dextran density gradient centrifugation. She found that the topmost fraction (lowest density) had the highest specific radioactivity of total hemoglobin. It contained a small percentage (7% of the total hemoglobin) of the major red

cell hemoglobin (Hb #1) and a large percentage (93% of the total hemoglobin) of the minor red cell hemoglobin (Hb #2). Also, in vivo administration of serum-bound ^{59}Fe followed by analyses of red cell hemoglobins confirmed that Hb #2 is associated with the "youngest" erythroid cells, while Hb #1 is associated with the "older" erythroid cells.]

In adult human erythroid cell, Reider et al. (1965) and Winslow et al. (1966) indicated that early erythroid cells synthesize more delta chains for human Hb A_2 than circulating reticulocytes. Moreover, the synthesis of delta chains is slower in the reticulocyte than that of alpha or beta chains. Therefore, the phenomenon of non-uniform biosynthesis of multiple hemoglobins is most likely to exist in adult human erythroid cells. Further proof of this theory can be obtained by the separation of human bone marrow cells with CCD or with density gradient centrifugation followed by the analysis of hemoglobin with IEF.

The Nature of Heterogeneity and the Synthesis of the Eight Rat Hemoglobins

In the human red blood cell, Hb F and Hb A exist in a single cell (Ingram, 1963), and gamma, delta, and beta chain genes are closely linked (Huisman et al., 1972). Garric et al. (1973) showed both hemoglobin A and hemoglobin C in Caprine ruminants are found in the same cells. The

eight (or six) rat hemoglobins, most likely exist in the same mature cell and are synthesized at different rates at different stages of maturation. Otherwise the patterns of hemoglobin from cells fractionated by CCD would have fixed distributions of the 8 hemoglobins compared with the pattern representing the development of young to old cells. In the whole animal, marrow and spleen tissues have identical hemoglobin patterns (i.e., 8 hemoglobins in the same proportion) and the same hemoglobin synthesis characteristics. Thus, there is no "two line" erythroid cell (no two separated clones of erythroid cells) in rat and maturation of the red cell is processed in only one erythropoietic organ. Hence, migration of cells from one erythropoietic organ to another (during maturation) does not occur in rats.

Eight rat marrow hemoglobins, isolated by IEF, have the same characteristics of absorption at 410 nm, 540 nm, and 575 nm. Generally, a multiplicity of hemoglobin bands in electrophoresis reflects a multiplicity of globin chains. Usually, all tetrameric hemoglobins studied have been shown to consist of two identical alpha chains and two identical non-alpha chains in adult inbred mammals. In most animals, if not all, the number of hemoglobins existing in adult mammalian RBC equal the number of types of alpha chain times the number of types of non-alpha chain. For instance, Kitchen (1974) reviewed that two alpha genes and one beta gene make two hemoglobins

in the RBC of the rabbit, the mouse, the deer, or the monkey (Macaca speciosa). One alpha gene and two non-alpha genes make two hemoglobins for RBC hemoglobins of the cat, the ape, the human, or New World primates. Huisman (1974) suggested that the heterogeneity of deer hemoglobins is the result of various combinations of two different alpha chain types and five beta chain types. Evidence has shown that the two alpha chains are the products of non-allelic structural Hb alpha-genes and 5 beta chains which are considered to be products of alleles of one structural Hb beta-gene. Thus,in deer with these Hb loci operative, two hemoglobins will be found in the animal which is homozygous for one of the five known Hb alleles, whereas four hemoglobins will be observed when the deer is heterozygous for two Hb beta-alleles. Totally, ten hemoglobins can be found in different Virginia white-tailed deer.

Garric et al. (1975) showed that in the rat there were 2 alpha chains (I_{α}, II_{α}) and 3 beta chains (I_{β}, II_{β}, III_{β}) and that these made six rat red blood cell hemoglobin fractions. The subunit composition of the six rat hemoglobins is Hb I ($II_{\alpha_2} III_{\beta_2}$), Hb II ($I_{\alpha_2} III_{\beta_2}$), Hb III ($II_{\alpha_2} II_{\beta_2}$), Hb IV ($I_{\alpha_2} II_{\beta_2}$), Hb V ($I_{\alpha_2} I_{\beta_2}$), and Hb VI ($II_{\alpha_2} I_{\beta_2}$). Their Hb numbers 1, 2, 3, 4, 5, and 6 correspond to Hb fractions I, II, III, VI, IV, and V in the present study. So far, only this tentative information is available and it indicates that the presence of two alpha chains probably represents non-allelic alpha loci

(i.e, an alpha-gene duplication). Also, the three beta chains may reflect a beta-gene triplication or they may be present due to some type of postsynthesis modification. E. Weiser (1976) found that there are three different alpha and three different beta chains for Hb III, IV and V using amino acid analysis of pure individual hemoglobin peptide chains through fingerprint mapping, which is based on 2-dimensional chromatographic and electrophoretic development of the hydrolysis products obtained by standardized degradation with trypsin. This result is possible since genetic evidence provides a strong indication for low-order reiteration of certain globin genes. For instance, in man there are two or more non-allelic loci for gamma-chains and at least two structural variants termed G_γ (glycine at position 136) and A_γ (alanine at position 136) (Schroeder et al., 1968). G_γ and A_γ genes are closely linked on the chromosome (Huisman et al., 1972). It would be possible for 3 beta chains and 2 alpha chains to make 6 hemoglobins in the erythrocyte as detected by IEF. If the above statements are correct, it can be assumed that 2 alpha genes are always active in the hemoglobin-synthesizing cell and that 3 beta gene have different activities in the different stages of cell. If the beta-structure genes were turned on, the order would be $I_\beta \longrightarrow III_\beta \longrightarrow II_\beta$ during the maturation. Therefore Hb V and Hb VI synthesis would be synthesized first

followed by Hb II and Hb I. Hb III and Hb IV synthesis would occur last. This theory can be proved by the data in Table 3 and 4, Figure 7.

From the developmental histroy of the organism (ontogeny), there is a shifting pattern associated with the hemoglobin system (i.e., in humans, Hb F switches to Hb A and Hb A_2 upon birth, all in a single cell). Certain alterations in the relative rates of hemoglobin synthesis were found in mouse embryonic hemoglobins (i.e., E_1, E_{11}, E_{111}) (Fantoni et al., 1969). The synthesis of all three embryonic hemoglobins can be detected by day 9 of gestation. Hbs E_1 and E_{111} synthesis is complete by day 11 whereas E_{11} is produced for at least 2 more days. In adult animals, sheep and goats begin to synthesize hemoglobin C ($\alpha_2 \beta_2^C$) instead of hemoglobin A ($\alpha_2 \beta_2^A$) in the same cell when the animals are made anemic (Nienhuis et al., 1974). These examples suggest that even within a single cell the production of individual globin chains may be under independent regulation of total amount of hemoglobin in one single cell, and may depend upon the interaction of these single genes. The nature of this control, whether at a level of transcription or translation of genetic information or some other locus such as differential cell-replication kinetics, has yet to be ascertained. Up to now, the molecular properties of Hb VII and Hb VIII are unknown. One possibility is that Hb VII and Hb VIII share one beta chain (designated as IV_β in this discussion) which is

different from I_β, II_β, III_β (as described by Garric et al., 1975), but contain different alpha chains (I_α, II_α). These IV_β hypothetical chains are synthesized before any other beta-chains (i.e., I_β, II_β, III_β) at the beginning of hemoglobinization and then IV_β synthesizing activity is turned off in the later erythroblast. There are also two possibilities for the extremely low concentration of Hb VII and Hb VIII in the red blood cell. One possiblity is that hemoglobin fraction VII and VIII are only produced in young cells (one or two cell divisions before the orthochromatic erythroblast), the other possibility is that hemoglobin synthesis (other than Hb VII and Hb VIII) in orthochromatic erythroblast and reticulocytes is very active and therefore dilutes the concentration of Hbs VII and VIII. A reason for the disappearance of Hbs VII and VIII may be that they, like most enzymes, are not as stable as the other hemoglobins and are therefore degraded after their synthesis ceases. Another possibility concerning the composition of Hb VII and Hb VIII is that they are made up of homotetramers such as III_{β_4} and I_{β_4} respectively and III_{β_4} and I_{β_4} would not be produced when the first alpha-chain was synthesized. The reason for this may be the imbalance of activity of alpha- and beta- chain synthesis at the beginning of hemoglobinization. Examples of this phenomenon can be found in the earliest human hemoglobin recognized in the 8-week embryo (Gower I) which is compsed of four identical ε-globin (ε_4) polypeptide

chains (Huehns et al., 1964). After the onset of alpha-chain synthesis, the cell forms hemoglobin Gower II ($\alpha_2 \varepsilon_2$). In alpha thalassemia, chemically normal beta and gamma chains, lacking alpha chains with which to combine, may have an abnormal grouping and so give rise to the abnormal forms of hemoglobin, Hb H (β_4) (Benesch, 1961) and Hb-Bart (γ_4) (Ohno, 1966) respectively. There is no evidence for α_4 in any mammals. Therefore the chance of I_{α_4} and II_{α_4} existence is very low. If Hb VII and Hb VIII are homotetramers of III_{β_4} and I_{β_4}, it can be assumed the order of globin gene turn on is (I_β and III_β) $\longrightarrow I_\alpha \longrightarrow II_\alpha \longrightarrow II_\beta$ which makes the order of hemoglobin turn on as: [Hb VII (III_{β_4}) + Hb VIII (I_{β_4})] $\longrightarrow$ [Hb V ($I_{\alpha_2} I_{\beta_2}$) + Hb II ($I_{\alpha_2} III_{\beta_2}$)] $\longrightarrow$ [Hb VI ($II_{\alpha_2} I_{\beta_2}$) + Hb I ($II_{\alpha_2} III_{\beta_2}$)] $\longrightarrow$ [Hb III ($II_{\alpha_2} II_{\beta_2}$) + Hb IV ($I_{\alpha_2} II_{\beta_2}$)]

This theory also agrees with data of Figure 7, Table 3 and 4. Certainly, the order of hemoglobin may be determined by an independent mechanism. The order of hemoglobin synthesis may be affected by the varying proportions of individual globin mRNAs and by the stability of each globin mRNA. A single regulatory mechanism may be responsible for the several kinds of shifts in hemoglobin synthesis although it is deemed unlikely from the present study. The actual situation can only be determined by isolation and identification of the individual globin genes for each of the eight components from cells of

different ages.

Possible Regulation of Hemoglobin Switching in Erythropoiesis

From the data in Figure 6 and 7 and Table 3, synthesis of Hb V occurs in "young" erythroid cells which then "switch" to synthesize Hb IV in "old" erythroid cells. By IEF analysis of rat RBC hemoglobins, the relative distribution of six hemoglobins is remarkably constant for many individuals and strains. Therefore we can conclude that the "final" concentration of specialized hemoglobins in each mature, differentiated cell is constant. After this concentration has been reached, the synthesis of specialized hemoglobins is discontinued. The in vitro labelling of reticulocyte-rich cells from phenylhydrazine-treated rats by ^{14}C-leucine (Table 4) resulted in unequal rates of six hemoglobins synthesis. It seems that Hb V reaches its "final" concentration earlier than that of Hb IV. These molecular transitions are clearly under genetic control, but the nature of the transition is not yet apparent. It is impossible for these to represent selective proliferation of hemoglobin, but it is obvious that synthesis of different types of hemoglobin at different stages of erythroid cell development occur at different rates (see Figure 6, 7) in rats. One determinant of these rates is the amount of pre-existing globin mRNA in the cell. The mechanisms of repression and activation of these genes remain unknown. The changes in non-histone protein

composition in the nucleus of avian erythroid cells occur at different stages of maturation (Ruiz-Carrillo et al., 1974). Stein et al. (1974) suggest that the phosphorylated non-histone chromosomal proteins play a key role in regulation of gene expression in the eukaryotic cell. If this is correct, hemoglobin would then be synthesized by different genes at different stages of maturation on the premise that the changes in cell function that depend on altered patterns of transcription will require changes in the interaction of regulatory proteins with DNA. Therefore, different globin mRNAs may be regulated by different non-histone nuclear protein before the cell becomes pyknotic. Ramirez et al. (1975) found the number of globin mRNA molecules to increase in mice fetal erythroid precursor cells, which contain little globin mRNA, to an average value of 1800 molecules per cell during 22 hours incubation. After 22 hours, the amount of globin mRNA remained constant. These findings suggest either that globin mRNA is synthesized principally in immature erythroblasts, or that by 22 hours, globin mRNA production and degradation are balanced. If the selective transcription of the structural genes for "switching" regulation of hemoglobin synthesis is the true mechanism for the rat hemoglobin switching, the complete mechanism of this transition should be in effect before the polychromatophilic erythroblast stage in development. After this stage, RNA synthesis ceases, and

the continuation of globin synthesis depends on pre-existing mRNA, tRNA, ribosomes and supernatant factors (Denton et al., 1973). For this reason, further separation of each stage of nucleated cells is essential. Qualitative and quantitative analysis of globin genes or specific activities of the globin genes from each stage of cell maturation is a means by which this theory can be explored.

There are several kinds of evidence which indicate that there are determinants of regulation of specific proteins present at the level of globin mRNA translation. For instance, in sheep (Baldy et al., 1972) or in rabbit (Lodish, 1974; McKeehan, 1974) there is a discrepancy between the relative amounts of alpha and beta globin mRNA and the synthesis of the alpha and beta globins. The production of two different chains, such as the alpha and beta chains, needs to be synchronized but the modulation of this synchronization is unknown. Garel (1974) suggests that tRNA specialization for protein synthesis can be both an aspect of differentiation and part of a functional adaptation of cells that synthesize special proteins for brief periods. Even the transport of the same amino acid may be affected by different tRNA levels. A deficiency in one type of tRNA can lead to impairment of one specific protein synthesis. This theory can be proved by the comparison of tRNA content to the amino acid component of the hemoglobins in erythroid cells at a certain stage.

For example, if the above hypothesis is correct, the tRNA content of rat reticulocytes is specialized for Hb IV and not for Hb V. It is not yet known which of these possibilities is the true cause. Moreover, it is possible that many small molecules and hormones are involved in hemoglobin "switching" in rat erythropoiesis.

Suggestions for Future Studies

1. The nature of the heterogeneity of rat hemoglobins: The components of each hemoglobin should be carefully studied and distinguished by the fingerprint and amino acid analyzer techniques.

2. Generality of non-uniform biosynthesis of multiple hemoglobins in other mammalian species: Use the same methods as we have studied in the rat and guinea pig in our laboratory to study other animals (i.e., sheep, cat, and human) with beta chain polymorphism and animals (i.e., deer, mouse, macaque, and rabbit) with alpha chain polymorphism. Lessard and Taketa (1969) found that a large number of cats showed the relative proportions of two hemoglobins ranged from about 1/1 to 9/1 with the 1/1, 2.3/1 and 9/1 phenotypes occurring most frequently in the general population. Kitchen (1974) found similar observations in stump-tail macaque hemoglobins. The ratios of two hemoglobins of the macaque were 60/40, 50/50, and 30/70 from three groups of animals. Therefore, it is necessary to study many species and to select the appropriate

animals to confirm the phenomenon of non-uniform biosynthesis of multiple hemoglobin.

3. To clarify the regulation of non-uniform biosynthesis of multiple hemoglobin:

If the presence of specific globin mRNA with the biosynthesis of particular globin results in non-uniform biosynthesis of multiple hemoglobins the youngest rat erythroid cell would then be capable of directing the synthesis of Hbs V, VII, VIII and not Hb IV. The method of rising a heterologous cell-free protein synthesizing system can be applied to estimate the amount of mRNA for each globin chain at various stages of erythrocyte development. If the non-uniform biosynthesis of multiple hemoglobin are caused by the concentration of a specific tRNA, then tRNA content and amino acid components of the hemoglobin should be measured. Investigation of the variable expressions of quantitative and qualitative control of animal hemoglobin production may serve as an ideal model for the study of the mechanisms of human hemoglobinopathies and hemoglobin biosynthesis.

4. Demonstration of committed stem cells in separated marrow cell fractions:

Till and McCulloch (1961) demonstrated a direct technique for the measurement of the number of committed stem cells in a bone marrow suspension capable of forming colonies in the spleen of lethally X-irradiated mice. By injecting

the CCD-separated marrow cells into the lethally X-irradiated animal, one may find the fraction(s) containing the committed stem cells for all different developing marrow cells.

5. Biochemical changes during erythrocyte differentiation: Additional studies with erythroid cells should look into sequence of biochemical changes in the activity of enzymes uniquely associated with the RBC and membrane components during erythroid cell differentiation and maturation.

SUMMARY

Rat bone marrow cells contain 8 different hemoglobins, two of which (Hb VII and VIII) are not detectable in circulating red blood cells.

The technique of countercurrent distribution (CCD), in buffered dextran-polyethylene glycol phase systems, has been used to separate rat bone marrow cell suspensions into fractions enriched in particular cell types, especially in the erythroid cell line. The increase in marrow erythroid cell partition (in surface charges) with the age of the cell is confirmed in these experiments. The two-polymer phase system does not damage the marrow cells' ability to incorporate serum-bound ^{59}Fe after the marrow cells are CCD. Quantitative changes in DNA, RNA, hemoglobin, and total protein for each cell from the various fractions have been characterized by chemical analysis, and correlated with the changing morphology of the erythroid cell during erythropoiesis. The most striking alterations in both quantitative and qualitative analysis of the rat hemoglobins are demonstrated to correlate with cells at particular stages during erythroid maturation, concomitant with the replacement of Hb V, VII, and VIII by the synthesis of other hemoglobins. This transition is accompanied by a quantitative increment in other hemoglobins. Fraction V serves as a marker for the youngest erythroid cell in rat whereas the synthesis of hemoglobin fraction IV serves as

a marker for the oldest hemoglobin-synthesizing erythroid cell. The content of hemoglobin VII and VIII provides an index for the earliest of the erythroid cells in rat marrow.

In vitro cohort labelling of total marrow cells with ^{59}Fe or ^{14}C-leucine resulted in equal specific radioactivities for Hbs I to VI whereas Hbs VII and VIII have higher specific radioactivities. It could be concluded that Hbs VII and VIII were synthesized at the "earliest" stage of erythroid cell development in cells with the highest protein synthesis activity. Marrow erythroid cells of rats were labelled, in vivo, with ^{59}Fe and the analytical results also lead to the conclusion that Hb V is associated with the "youngest" erythroid cells whereas Hb IV is associated mostly with the "oldest" erythroid cells. This was substantiated by the finding that in vitro labelling with ^{14}C-leucine of reticulocyte-rich cells from phenylhydrazine-treated rats resulted in unequal specific radioactivities; Hb IV had the highest specific radioactivity whereas Hb V had the lowest.

All these findings are suggestive of non-uniform biosynthesis of the rat multiple hemoglobins during erythroid cell development.

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