Mouse Hemoglobin from Bone Marrow, Spleen, and Peripheral Blood Cells[1] (34786)

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Mouse hemoglobin has been classified according to its electrophoretic fractionation as single (homogeneous) or diffuse (heterogeneous). The hemoglobin differences were directly referable to the strain of mouse examined (1–9). In 1962 Hutton *et al.* (10, 11) reported that the hemoglobin from circulating erythrocytes of mice with diffuse hemoglobin was composed of different β chains and a single type of α chain. Popp demonstrated through solubility studies that more than one α chain could be present in hemoglobins classified as the single hemoglobin type (12, 13). This was confirmed by chemical characterization of the α chain (14, 15). However, the amino acid changes in the α chain so far described are all of equivalent ionic charge and have no effect upon electrophoretic mobility. Both electrophoresis and ultracentrifugation gave evidence for aggregation of mouse hemoglobin upon aging *in vitro* (16, 17). In 1964, Riggs *et al.* (18) demonstrated that polymerization of the diffuse hemoglobin in the circulating erythrocyte resulted in a 7S molecule composed of eight polypeptide chains linked through disulfide bonds between β chains. Later Bonaventura and Riggs showed that this disulfide bond between the β chains occurred through cysteine at residue 13 on the β chains (19). Morton (17) suggested that a changing equilibrium between subunits was responsible for the pattern changes associated with *in vitro* aging of diffuse hemoglobin.

All of the above-reported studies and many others have used the circulating erythrocyte in the peripheral blood as a source of hemoglobin. The study reported herein has compared the hemoglobin from the circulating erythrocyte to the benzidine-positive material found in the spleen and bone marrow. A heretofore unsuspected difference in these fractions was found. The benzidine-positive material from the spleen or bone marrow of the mouse with diffuse hemoglobin separates into two fractions upon starch-gel electrophoresis instead of the four fractions of hemoglobin found in the peripheral blood of the same mouse. Only one electrophoretic fraction of benzidine-positive material or hemoglobin is present in the spleen, bone marrow, and peripheral blood of the mouse with hemoglobin classified as single hemoglobin. Phenylhydrazine-induced anemia with subsequent reticulocytosis and increase in hematocrit is accompanied by changes in the electrophoretically fractionated benzidine-positive materials in spleen and bone marrow and the hemoglobin fractions in peripheral blood.

Materials and Methods. Mice. Adult male mice weighing 27–30 g from an inbred colony of Swiss mice, designated Tenn-Swiss were examined to characterize the hemoglobin found in their peripheral blood, bone marrow, and spleen. Peripheral blood was obtained from ether-anesthetized animals by exsanguination after severing of the brachial artery in the right axillary area. The cells were collected in 0.85% sodium chloride (NaCl). After exsanguination the spleen was removed, weighed, and placed in cold 0.85% NaCl. The spleen tissue was teased and minced to free cells. The long bones from all limbs were removed, cleaned of soft tissue, washed in cold 0.85% NaCl, and opened by cutting off the epiphyses of the bones. The bone marrow was expelled from the bone by flushing in a

[1] This investigation was supported in part by Grant HE-06627, National Heart Institute, and Grant AM 13248, National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service and by Grant 223001 Community Support for Undergraduate College Students.

stream of 0.85% NaCl under pressure. When the liver was examined, it was removed, minced, and the cells freed in 0.85% NaCl. Cells from the peripheral blood, bone marrow, spleen, and liver were washed three times in 0.85% NaCl and cell hemolyzates were prepared by a modified Drabkin technique (20). The cells were ruptured in cyanmethemoglobin reagent (21) and toluene and centrifuged at 10,000 rpm for 20 min in the cold. The clear red cell hemolyzate was aspirated and filtered through packed glass wool. Pooled peripheral blood, pooled spleen, pooled bone marrow, and pooled liver from 5–15 mice was used in each experiment. The concentration of cyanmethemoglobin was measured (21) and adjusted to 0.3 g per 100 ml. Immediately, 25 μl of the 0.3 g/100 ml hemolyzate was fractioned by vertical starch-gel electrophoresis (22); 12.8% starch, pH 8.6, Tris–EDTA–boric acid buffer (23), 17 hr, 250 V, and 15 mA. The gels were sliced and stained with a benzidine stain.

In addition, three genetically distinct groups of adult mice (30 g) were studied as described above. One group consisted of C57BL/Cum mice. Two other groups were bred and described by Popp (24, 25). These animals were developed from a cross of C57BL/Cum and BALB/cJ mice, then selected and bred to maintain the hemoglobins characterized by $\alpha^{BALB/c}\beta^{C57BL/Cum}$ or $Hba^{2,3}Hbb^{s}$ (with agouti coat color) or the hemoglobin $\alpha^{C57BL/Cum}\beta^{BALB/c}$ or $Hba^{1}Hbb^{d}$ (with white coat color) (26).

Phenylhydrazine Anemia. Tenn-Swiss mice were rendered anemic by injections of phenylhydrazine (1.6 mg/30 g body weight) according to Shadduck *et al.* (27). The phenylhydrazine in 0.85% NaCl was injected subcutaneously on Day 0, Day 1, and Day 3. Development of the anemia was followed by using microhematocrit determinations and reticulocyte counts. Sufficient blood for these studies was obtained from the periorbital capillary plexus. The reticulocytes were stained by mixing equal volumes of new methylene blue and blood, incubating at room temperature for 20 min, and then visualized in a dried blood smear. The phenylhydrazine was reacted with the benzidine stain to see if such a mixture would give a positve benzidine test. The test was negative. No color formation was observed in the mixture.

Results. Normal Tenn-Swiss mice. The Tenn-Swiss mouse hemoglobin from peripheral blood upon fractionation by starch gel electrophoresis separates into four bands in agreement with the results of Rosa *et al.* (6) and others. Thus Swiss mouse hemoglobin is a diffuse hemoglobin according to mouse hemoglobin classification. The four hemoglobin fractions are visualized in Fig. 1. The numbering system used herein was used first in 1966 by this laboratory (28) and will be retained for purposes of clarity. The benzidine-positive materials obtained from bone marrow or spleen cells from the same animals fractionated into only two bands (Fig. 1). The electrophoretic mobility of the slower moving (more cathodal) of these bands does not appear to be identical with the mobility of band III from peripheral blood. Band III of peripheral blood contains all the soluble tryptic peptides found in the total cell hemolyzate (28). Therefore, hemoglobin fraction III is considered to be the polymeric hemoglobin described in the peripheral blood of the mouse with diffuse hemoglobin. There is less of the most anodal fraction in peripheral blood than there is in spleen or bone marrow. If the mice were not exsanguinated before the spleen was removed, the electrophoretic fractionation of spleen benzidine-positive material showed contamination with enough peripheral blood to give faint bands of hemoglobins I and II and slight broadening of hemoglobin III band.

C57BL/Cum mice. The C57BL/Cum mouse is known to have a single band of hemoglobin in peripheral blood (13). The hemoglobin of this strain contains one type of α chain and one type of β chain when the tetrameric hemoglobin is characterized. The C57BL/Cum mouse has only a single hemoglobin fraction in the cell hemolyzate of peripheral blood which has the same electrophoretic mobility as that of the single fraction of benzidine-positive material from the spleen or bone marrow (Fig. 2).

$\alpha^{BALB/c}\beta^{C57BL/Cum}$mice. The hemoglobin

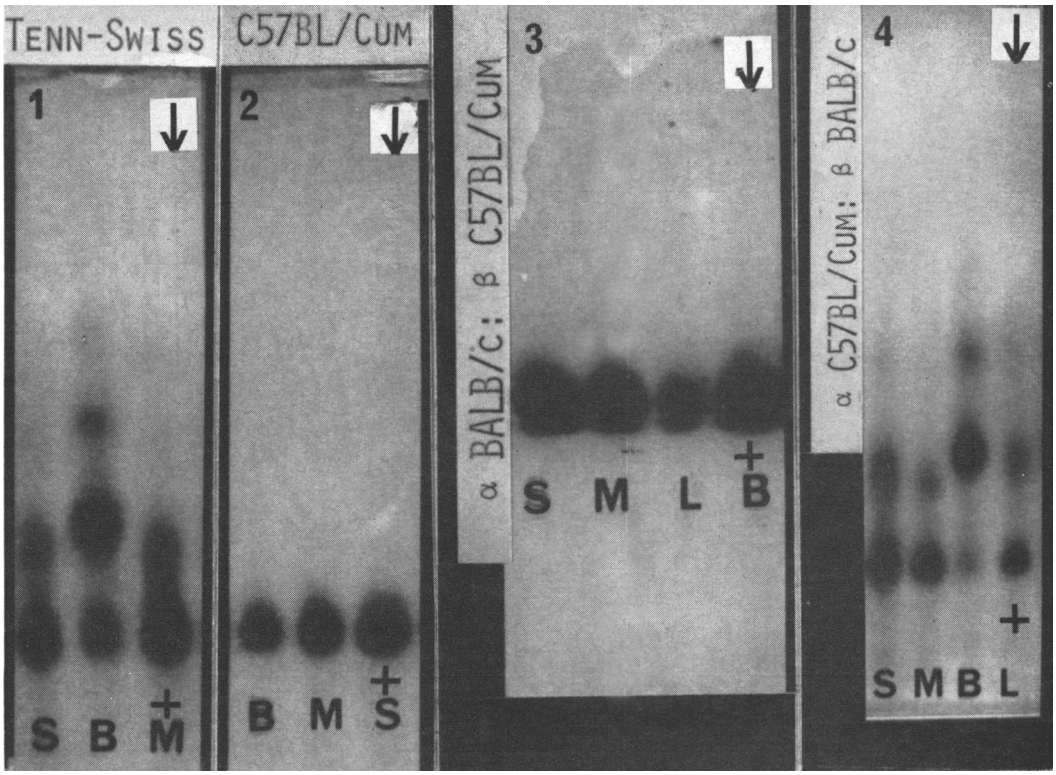

FIG. 1. Tenn-Swiss mice cell hemolyzate from S (spleen), B (peripheral blood), and M (bone marrow) fractionated by vertical starch-gel electrophoresis, 12.8% starch, pH 8.6, Tris–EDTA–boric acid buffer, 17 hr, 250 V, 15 mA, 0.025 ml of 0.3 g/100 ml hemoglobin. Stained with benzidine stain. The peripheral blood fractions are numbered sequentially I through IV. Fraction I being most cathodal and fraction IV being most anodal.

FIG. 2. Starch-gel electrophoretic fractionation of cell hemolyzate from S (spleen), M (bone marrow), and B (peripheral blood) of C57BL/Cum mice. Experimental conditions described in Fig. 1.

FIG. 3. Starch-gel electrophoretic fractionation of cell hemolyzate from S (spleen), M (bone marrow), L (liver), and B (peripheral blood) of $\alpha^{BALB/c}\beta^{C57BL/Cum}$ mice. Experimental conditions described in Fig. 1.

FIG. 4. Starch-gel electrophoretic fractionation of cell hemolyzate from S (spleen), M (bone marrow), B (peripheral blood), and L (liver) of $\alpha^{C57BL/Cum}\beta^{BALB/c}$ mice. Experimental conditions described in Fig. 1.

of the agouti coat-colored mice has the α chain structural genes of the BALB/c parent and the β structural genes of the C57BL/Cum parent. Upon electrophoresis of hemolyzate from peripheral blood, a single hemoglobin band of apparent identical mobility to the benzidine-positive material fractionated from bone marrow, spleen, and liver was seen (Fig. 3).

$\alpha^{C57BL/Cum}\beta^{BALB/c}$mice. White mice having the α chain of the C57BL/Cum parent and the β chain from the BALB/c parent have hemoglobin in the peripheral blood which fractionated into the typical bands found in the mouse with diffuse hemoglobin. These four hemoglobin fractions migrate in a similar manner as those seen in the Tenn-Swiss mouse. The spleen and bone marrow benzidine-positive materials divide into two bands, the more cathodal being different from the major band in the peripheral blood (Fig. 4). The benzidine-positive fractions from the

liver appear to be similar to the peripheral blood fractions except that the most anodal fraction is slightly increased (Fig. 4). The two α chains of the BALB/c mice are electrophoretically indistinguishable, the difference being a serine residue or a threonine residue at position 68 (14). C57BL/Cum mice have a single α chain and a single β chain. Animals whose hemoglobin has a single β chain and a single α chain or electrophoretically indistinguishable α chains have only one hemoglobin fraction in their peripheral blood and one benzidine-positive fraction in bone marrow and spleen. However, mice whose hemoglobin has two BALB/c β chains and the single C57BL/Cum α chain have four hemoglobin fractions in their peripheral blood and two benzidine-positive fractions in their spleen and bone marrow. The same type of fractionation was found in the Tenn-Swiss mice.

Phenylhydrazine-induced anemia in Tenn-Swiss mice. Male Tenn-Swiss mice were rendered anemic with phenylhydrazine and divided into four to five animals per group. The development of the anemia is reported in Fig. 5. The reticulocyte and hematocrit values are averages of measurements from blood obtained on Days 4, 5, or 6 from each animal in the groups that were killed on Days 10 and 12. Thereby each of these mice was bled (0.2–0.3 ml) once during the course of the phenylhydrazine-induced anemia. Electrophoretic fractionation of unpooled blood from the individual bleedings on Days 4, 5, and 6 showed similar patterns of four hemoglobin bands in each hemolyzate. Although the cell hemolyzate was prepared in the same manner each day, on Day 6 hemoglobin precipitated from the filtered hemolyzate. Figure 6 depicts the electrophoretic fractionation of hemoglobins in the peripheral blood, and benzidine-positive material from spleen and bone marrow of normal mice, and mice with phenylhydrazine-induced anemia at Day

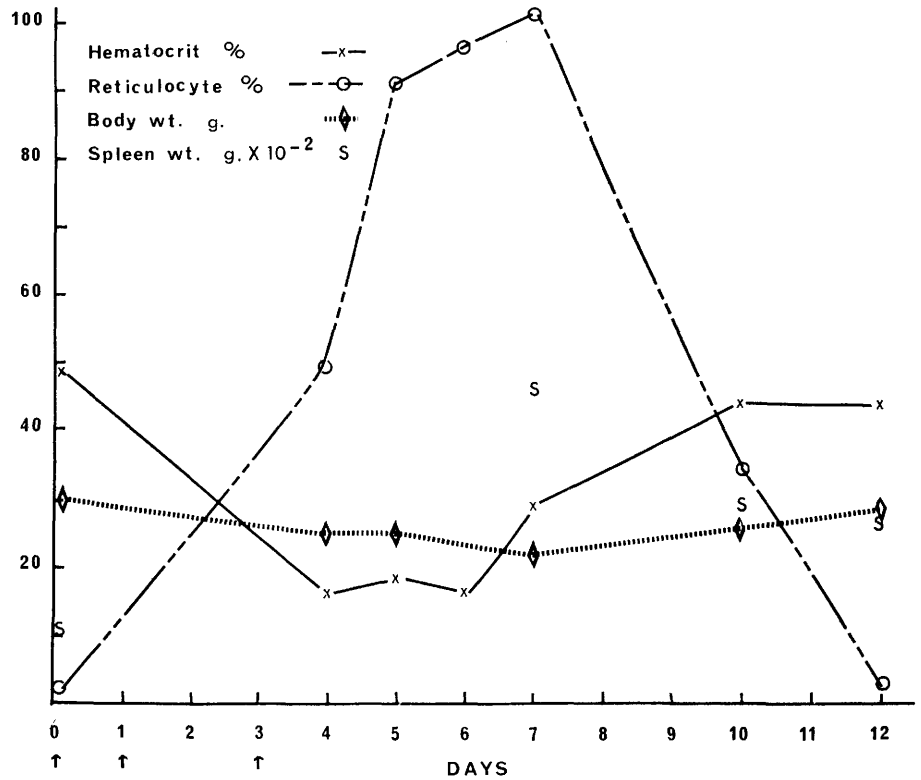

FIG. 5. Development of anemia in Tenn-Swiss mice after injections of phenylhydrazine (1.6 mg/30 g body weight) subcutaneously on Days 0, 1, and 3.

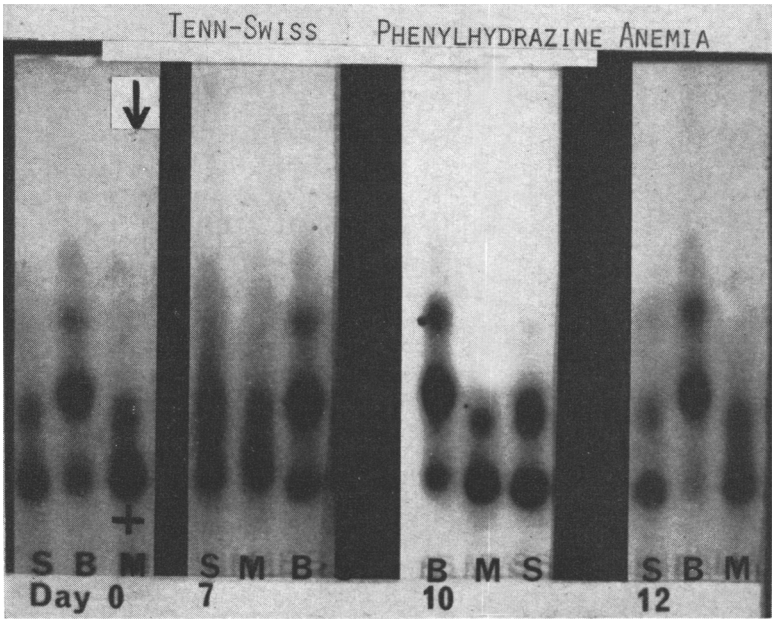

FIG. 6. Starch-gel electrophoretic fractionation of cell hemolyzate from S (spleen), B (peripheral blood), and M (bone marrow) of Tenn-Swiss mice on Days 0, 7, 10, and 12 of phenylhydrazine anemia. Experimental conditions described in Fig. 1.

7, 10, and 12. The most marked change is seen in the most anodal hemoglobin (Hb IV) of peripheral blood, which appears increased at the peak of reticulocytosis on Day 7 and markedly decreased as these cells age by Day 12. On Day 7, the spleen has a third fraction with an intermediate mobility between peripheral blood bands III and II. This is clearly seen at 8 days and is diminished at Day 12. The more cathodal of the two benzidine-positive bands regularly seen in spleen and bone marrow appears to be increased at Day 7 and this spleen fraction is markedly different at Day 10. Therefore, it is apparent that induced stimulation and change in the erythrocytic compartments of spleen and bone marrow are also accompanied by changes in the electrophoretically different benzindine-positive materials found in spleen and bone marrow (Figs. 5 and 6).

Discussion. Previous observations in this laboratory (28) indicated that under certain conditions stimulatory for erythropoiesis, the relative amounts of hemoglobin in the four fractions of diffuse mouse hemoglobin seemed to vary. Both spleen and bone marrow are sites of erythropoiesis in the adult mouse (29). Therefore, it seemed likely that any changes in hemoglobin fractions due to stimulation of erythropoiesis might be seen more readily in hemolyzates from these sources. Since precautions were maintained so that few if any of the changes seen could be due to *in vitro* aging, all of the electrophoretic fractions may reflect the state of the hemoglobin *in vivo*. Chemical characterization of the benzidine-positive fractions from spleen and bone marrow is necessary for positive identification as hemoglobin. This characterization is in process.

Summary. Upon fractionation by starch-gel electrophoresis:

(1) In the mouse with the single-type hemoglobin (C57BL/Cum) or the mouse with the β chain component being from the single-electrophoretic benzidine-positive fraction of the same mobility as that of hemoglobin from the peripheral blood was found in cell hemolyzates of bone marrow and spleen.

(2) In the mouse with diffuse type hemoglobin (Tenn-Swiss) or with the β chain com-

ponent from the diffuse type of hemoglobin ($\alpha^{C57BL/Cum}\beta^{BALB/c}$), the previously reported four hemoglobin fractions were seen in the peripheral blood; however, only two benzidine-positive fractions were found in the spleen and bone marrow.

Phenylhydrazine-induced anemia is accompanied by changes in the electrophoretically fractionated benzidine-positive materials from spleen and bone marrow and in the hemoglobin fractions from the peripheral blood.

The author thanks Dr. Raymond A. Popp, Biology Division, Oak Ridge National Laboratory for the special strains of mice (C57BL/Cum, $\alpha^{BALB/c}\beta^{C57BL/Cum}$, and $\alpha^{C57BL/Cum}\beta^{BALB/c}$, which he provided for this study.

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Received Nov. 21, 1969. P.S.E.B.M., 1970, Vol. 134.