Globin Composition and Synthesis of Hemoglobins in Developing Fetal Mice Erythroid Cells

Abstract. *Fetal mouse erythropoiesis proceeds initially in yolk-sac blood islands (8 to 12 days) and, subsequently, in liver (12 to at least 16 days). Yolk-sac cells synthesize three hemoglobins, Hb E_I, Hb E_{II} and Hb E_{III}. Hb E_I has x- and y-globin chains; Hb E_{II} has α and y; HB E_{III}, α and z. No detectable β-globin is formed in these cells. Liver erythroid cells form only adult hemoglobin, composed of α- and β-chains.*

Erythroid cells in fetal mice provide an excellent system for the study of the control of protein synthesis during cell differentiation. Studies in which starch-gel (*1*) or polyacrylamide-gel disc electrophoresis (*2*) is used to separate hemoglobins have resolved three or four components in red cells from fetal mice (strain C57B1/6J), only one of which persists in the adult mouse. During the development of the fetal mouse, which has a gestation period of 21 days, erythropoiesis occurs initially in yolk-sac blood islands (8th to 12th day) and, subsequently, in the fetal liver (12th to at least the 17th day) and in bone marrow (after day 16) (*3*). Previous investigations have shown a correlation between the disappearance from the peripheral blood of fetal mice of embryonic type hemoglobins and the decrease in erythroid cells derived from yolk-sac blood islands (*1*). More recently, studies with partially purified preparations of erythroid cells suggested that yolk-sac erythroid cells form predominantly embryonic hemoglobins, while liver erythroid cells synthesize predominantly adult hemoglobin (*2*). A sequential synthesis of different types of hemoglobins during fetal and postnatal development has also been reported for man, the tadpole, the chick, the duck, and the elephant (*4*).

The hemoglobins of man have been the best characterized (*5*). The three types of human hemoglobin, embryonic, fetal, and adult, differ in only one of the two pairs of globin chains. The composition of one embryonic hemoglobin (Gower II) is $\alpha_2\epsilon_2$; of fetal hemoglobin, $\alpha_2\gamma_2$; and of the major component of normal adult hemoglobin, $\alpha_2\beta_2$. A second embryonic hemoglobin (Gower I) is believed to be composed solely of ϵ-chains. There is no information on whether, in human fetuses, embryonic hemoglobin is formed in the same or different erythroid cells as fetal and adult hemoglobins. There has been no previous report on the globin chains composing embryonic hemoglobins of the mouse.

We have examined the composing embryonic and adult types of hemoglobin in C57B1/6J mice. In addition, our data establish that during fetal mouse development embryonic types of hemoglobin are formed in yolk-sac erythroid cells, while adult hemoglobin is synthesized in erythroid cells differentiating in the liver.

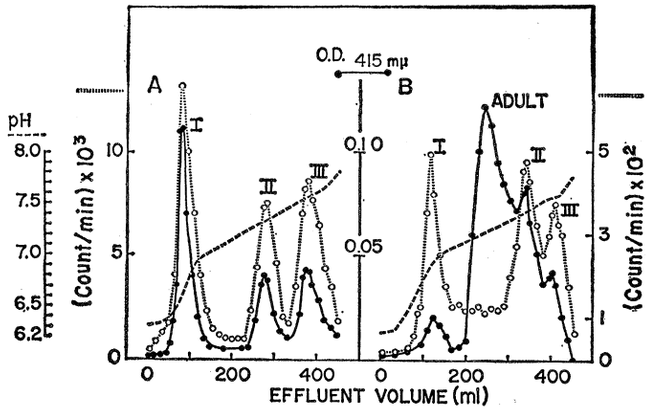
Mice of C57B1/6J inbred strain were purchased from the Jackson Laboratory. Litters of known gestation period were obtained by the method of Southard *et al.* (*6*). The methods for preparing yolk-sac erythroid cells and liver erythroid cells have been previously described (*3*). Erythroid cells (5×10^8 to 10×10^8) were incubated for 1 hour at 37°C with 2.5 μc of uniformly labeled ^{14}C-L-valine (215 μc/μmole); other conditions of incubation being as described elsewhere (*2*).

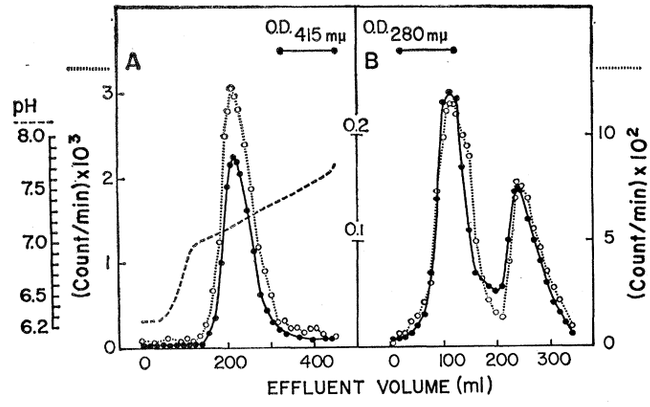
After incubation, the cells were lysed by being frozen and thawed three times in the presence of 0.001*M* Na_2HPO_4 (*p*H 7.4). The hemolyzate was centrifuged at 20,000*g* for 15 minutes, and the pellet was discarded. The clear supernatant fluid was bubbled with carbon monoxide to convert the hemoglobins to carbon monoxide hemoglobins. This solution was dialyzed overnight at 4°C against the developing buffer used for the column chromatography. The hemoglobins were separated by carboxymethyl cellulose (CMC) chromatography with a *p*H gradient (*7*). The dialyzed solution, containing 3 to 6 mg of protein per milliliter, was placed on a column (1 cm by 15 cm) of CMC previously equilibrated at *p*H 6.3 and washed with 100 ml of 0.01*M* Na_2HPO_4 (*p*H 6.3). Elution was performed with a gradient from *p*H 6.3 to *p*H 7.8 of phosphate buffer of constant molarity (0.01*M* Na_2HPO_4). The *p*H gradient was adjusted so that a change in *p*H from 6.3 to 7.0 occurred within the first 50 ml of the eluate, and a change in *p*H from 7.0 to 7.8 occurred in the next 100 ml of eluate. Elution of the column was performed at 18°C with a flow rate of 1 ml/min. Fractions of the eluate were collected and analyzed for optical density at 415 mμ and for radioactivity (*2*). The fractions containing each hemoglobin were pooled and adjusted to *p*H 7.3 for the preparation of globin. Globin was prepared from the total hemolyzate and from these isolated hemoglobin fractions by the method of precipitation with acid acetone (*8*). Globin chains were separated on CMC columns prepared in 8*M* urea and 0.05*M* 2-mercaptoethanol (*9*). A globin sample containing about 30 mg of protein was placed on a CMC column (1 cm by 20 cm) and eluted with a Na_2HPO_4 concentration gradient (0.01*M* to 0.025*M* at *p*H 6.8. The elution was performed at 18°C temperature with a flow rate of 1 ml/min. Fractions of the eluate were collected and analyzed for optical density at 280 mμ and for radioactivity (*2*).

Erythroid cells of yolk-sac origin are the only type present in peripheral blood of fetal mice at 11 days of gestation (*2, 3*). Chromatography of hemolyzates prepared from yolk-sac erythroid cells show three types of hemoglobin (Fig. 1A). There is ^{14}C-valine incorporated into each hemoglobin, indicating each is formed in these cells. These three hemoglobins differ from adult hemoglobin in the pattern of elution from the CMC column (Fig. 1B). These embryonic hemoglobins are designated as Hb E_I, Hb E_{II}, and Hb E_{III}, corresponding to the order of elution from this column. In these experiments, no detectable hemoglobin of adult type appears to be synthesized by yolk-sac erythroid cells.

Chromatographic analysis of the globin chains in the total hemolyzate of yolk-sac erythroid cells reveals four types, designated α, x, y, and z, of which only the α-chain corresponds to a globin present in adult hemoglobin (Fig. 2A). These data suggest that the β-chain of adult globin is not formed in yolk-sac erythroid cells. Each embryonic hemoglobin contains two types of globin chains. Hb E_I contains x- and y-chains (Fig. 2B); Hb E_{II} contains α- and y-chains (Fig. 2C); and Hb E_{III} contains α- and z-chains (Fig. 2D). Hb E_I does not appear to contain a globin chain corresponding to one of the adult globins.

Erythroid cells developing in fetal liver contain and synthesize a hemoglobin which is indistinguishable, by CMC column chromatography, from adult hemoglobin (Fig. 3A). In addition, the chromatographic behavior of the globin chains of the hemoglobin formed in liver erythroid cells is indistinguishable from that of adult hemoglobin (Fig. 3B). Further evidence





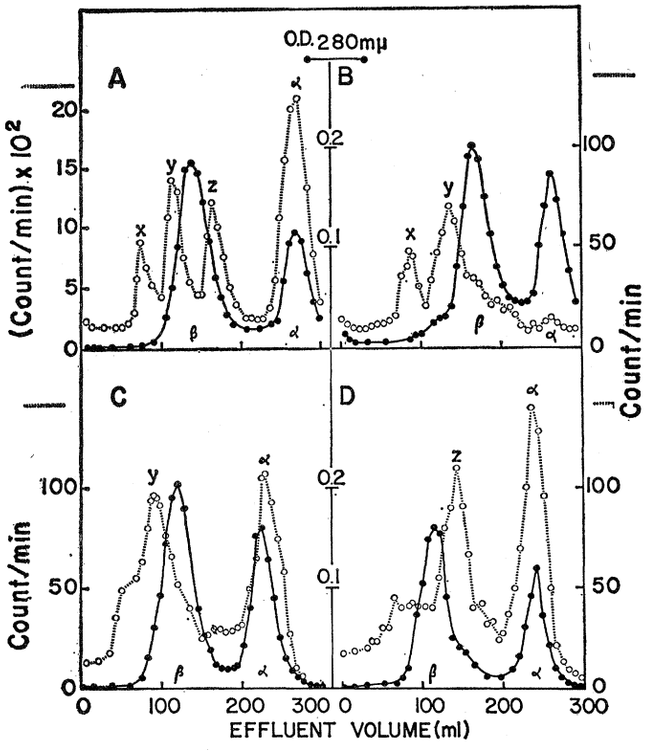


Fig. 1 (above left). Chromatography of the hemoglobins synthesized in yolk-sac erythroid cells. Hemolyzates were prepared from the cells obtained from 11-day fetal mice which were incubated with ^{14}C-valine as described in the text. The details of the chromatographic procedure using CMC are indicated in the text. The elution pattern of radioactivity (○) and optical density (●) without added carrier are shown in (A), and with added unlabeled hemolyzate prepared from the erythroid cells of adult mice in (B). (I) Hb E_I, (II) Hb E_{II}, (III) Hb E_{III}. Fig. 2 (left). Chromatography of the globins synthesized in yolk-sac cells. Globin was prepared from the hemolyzates of cells obtained from 11-day-old fetal mice which were incubated with ^{14}C-valine as described in the text. Adult mouse hemoglobin was added as carrier and as a marker in each of these preparations. The details of the CMC chromatographic procedure are indicated in the text. (○) Radioactivity; (●) optical density at 415 mμ. (A) Globin prepared from the total hemolyzate of yolk-sac erythroid cells. (B) Globin prepared from Hb E_I isolated by chromatography as indicated in Fig. 1. (C) Globin prepared from Hb E_{II} as shown in Fig. 1. (D) Globin prepared from Hb E_{III} as shown in Fig. 1. The β- and α-globin chains of adult mouse hemoglobin are denoted in the lower part of each frame, while the embryonic globin chains, α, x, y, and z, are denoted on the upper part of each frame. Fig. 3 (above right). Chromatography of hemoglobin (A) and globin (B) obtained from liver erythroid cells incubated with ^{14}C-valine. The procedures are described in the text. (○) Radioactivity; (●) optical density. Adult hemoglobin was added as marker.

suggesting that the hemoglobin formed in fetal liver erythroid cells is of adult type is that ^{14}C-valine incorporation into α- and β-chains is in a ratio of 1.0 to 1.4, which is in agreement with the ratio of the valine content of these two globin chains as determined by Popp (*10*).

Our study indicates that three types of embryonic hemoglobin are formed by yolk-sac erythroid cells. Hb E_{II} and Hb E_{III} differ from each other and from adult hemoglobin in the nature of the β-type globin, all containing α-globin. This is analogous to the differences in the composition of human embryonic, fetal, and adult hemoglobins. It is possible that one of the embryonic globin chains differs from another only by the presence of an NH_2-terminal acetyl group, as reported for human fetal hemoglobin and tadpole and bullfrog hemoglobin (*11*). Thus, the x- and y-globin of Hb E_I may differ only in one having an NH_2-terminal acetyl group, and this hemoglobin could then be analogous to Hb Barts ($\gamma_2\gamma_2^{acetyl}$) described for human fetuses (*11*). Further characterization of the mouse embryonic globin chains is required to resolve these possibilities.

Our findings indicate that in yolk-sac erythroid cells possibly as many as four types of globins are formed, only one of which, α-globin, is synthesized by fetal liver erythroid cells and by adult erythroid cells. As the site of erythropoiesis in developing fetal mice shifts from the yolk-sac blood islands to the liver, the type of hemoglobin formed changes to adult hemoglobin, and there is no detectable formation of embryonic hemoglobins. The differences in types of hemoglobins formed in yolk-sac and liver erythroid cells presumably reflects differences in gene transcription or mRNA translation. These two erythroid cell lines could develop from different precursor cells in the yolk-sac blood islands and in the liver. Alternatively, as gestation proceeds, erythroid cells of yolk-sac blood islands could seed the developing liver, and the change in the types of globin formed would reflect a response to the new environment.

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Collagen-Derived Membrane: Corneal Implantation

Abstract. *Behavior of membranes derived from collagen was investigated in rabbit corneas. Disks of the membrane were placed between the lamellae of corneas which were then examined grossly, biomicroscopically, and histologically. The membranes remained clear, and almost no reaction or evidence of reabsorption was seen during an observation period of 6 months. These characteristics make the material potentially useful for heterotransplantation in the cornea.*

Synthetic polymers have attracted considerable attention in medicine, especially in ophthalmology. Only a few of these synthetics, however, have been found with the inertness and other physical properties needed for use in the eye. The search for new materials has overlooked biopolymers, largely because understanding of their molecular biology has been limited, and also because of such related problems as availability, plasticity, and tissue reaction.

New information about the collagen molecule has, however, permitted the development of a collagen-derived biopolymer with unique properties (*1–4*). This transparent material has a refractive index similar to that of the corneal stroma and is a polyelectrolyte which can be molded into any form desired. Binding heparin to it will produce a nonclotting surface. Pore aperture can be varied to selectively permit or deny passage of different sized molecules. Before any practical use can be made of a new biological substance, its behavior in vivo must be studied. This material was studied by means of its implantation into the cornea.

The preparation of the collagen biopolymer has been described elsewhere (*1, 4*). Collagen from calfskin was used exclusively, although any tissue rich in this protein is a possible source.

Enzyme-treated collagen was polymerized from solution into transparent membranes. The surface charge was unaltered, and the pore size in the membranes used was 30 to 50 Å. A similar membrane used for hemodialysis was shown to permit relatively free passage of water, gases, Na^+, and K^+ (*1*).

Adult albino rabbits (2 to 3 kg) were used throughout. The animals were anesthetized with sodium pentobarbital (30 mg/kg of body weight) injected intravenously. Tetracaine-HCl (0.5 percent) was used topically for additional anesthesia.

Collagen membranes were sterilized by exposure to dry heat at 120°C for 2 hours. Disks 5 mm in diameter were removed from the membrane with a trephine and placed intralamellarly into 28 rabbit corneas. Lids were left open, neosporin ointment was applied immediately after surgery, and some animals received antibiotics prophylactically.

All eyes were examined grossly, and most biomicroscopically, at frequent intervals for signs of reaction, such as perilimbal vascular dilation, corneal edema and vascularization, hyperemia of the iris, and cells and flare in the anterior chamber. Animals were killed at regular intervals, and the eyes were fixed in 10 percent neutral formalin. Histologic sections were made of corneas which were then stained with hematoxylin and eosin, Hale's colloidal iron, and periodic acid–Schiff reagent.

In 25 eyes, the only visible reaction after the implantation was a mild dilation of the perilimbal vessels lasting from the 1st to the 3rd day. There was no iris hyperemia, corneal edema, or vascularization. There was no reaction at the interface of cornea and membrane. Throughout the period of observation, the membranes maintained their transparency and original appearance. There were no corneal erosions and no extrusions of the membrane.

In three eyes, there was a violent reaction marked by corneal infiltration and vascularization which did not respond to antibiotics. After about 3 weeks, small, grey punctate lesions were seen on the surface of the membranes in all three cases. Microscopic examination showed that they were fungus.

Six animals were killed at weekly intervals, and six were killed at monthly intervals. The corneal stroma appeared normal in all tissue sections, with the exception of the stroma posterior to the membranes which seemed somewhat edematous after 4 months. At no time were more than a few cells seen near the membrane. These cells appeared to be macrophages and fibroblasts, with an occasional mononucleocyte. The membranes appeared unchanged during the 6-month observation period. No cellular invasion or repopulation was seen. Membranes in place for several months stained a pale blue with Hale's colloidal iron stain, thus suggesting the presence of host acid mucopolysaccharide in the membrane.

Collagen materials implanted into the cornea have always provoked a cellular response and are eventually reabsorbed. The behavior of plain catgut is well known (*5*). This familiar material, prepared from ovine and bovine intestinal collagen, is well tolerated but produces a much greater cellular response than collagen membranes do. Other collagen-rich implants, such as rat tail tendon (*6*), cartilage (*7*), and dissolved collagen fiber (*8*), have provoked tissue responses of varying degree, but always significantly greater than those caused by the collagen membranes used here.

Collagen placed in the cornea has inevitably been reabsorbed, but at a highly variable rate. Payrau polymerized collagen in the living rabbit cornea and found no evidence of it after 24 hours (*8*). Plain catgut is reabsorbed in a few days (*9*), and cartilage is resorbed over a period of months (*7*).

It is not definitely known why this collagen membrane when implanted