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**Research Article**

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# Postnatal Fetal and Adult Hemoglobin Synthesis in Early Preterm Newborn Infants

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**ABSTRACT** Studies were carried out during the postnatal period in infants born at or before the 32nd wk of gestation to determine the proportion of fetal hemoglobin (Hb F) and adult hemoglobin (Hb A) being synthesized, and to compare these studies to those previously reported on at birth from normal newborn infants 25–43 wk gestation. When the preterm infants reached the postconceptional age corresponding to term, their Hb A and Hb F synthesis was compared to a group of newborn infants at term. 53 blood samples from 25 preterm and 11 full-term infants were incubated in an amino acid mixture containing [<sup>14</sup>C]leucine, and column-chromatographed on DEAE-Sephadex for separation of Hb F and Hb A fractions. The completeness of the DEAE-Sephadex separation of Hb F and Hb A was confirmed by globin chain chromatography with the use of carboxymethylcellulose. The rate of transition from Hb F to Hb A synthesis postnatally in the preterm infants resembled that reflecting the *in utero* transition. At the postconceptional age corresponding to term, there was no difference in the relative amounts of Hb F and Hb A being synthesized by the preterm infants and by the term infants. The birth process did not alter the rate of transition from Hb F to Hb A.

## INTRODUCTION

During intrauterine life, there is an orderly transition from fetal hemoglobin (Hb F) to adult hemoglobin (Hb A) synthesis. The percent of Hb F synthesized is high during early gestation and decreases with increasing gestational age (1). There is no information available regarding environmental effects on the postnatal transition from Hb F to Hb A synthesis in infants born prematurely. Does a premature exposure to extrauterine life affect this orderly transition?

The present study was carried out with two goals in mind: first, to determine serially Hb F and Hb A syn-

thesis postnatally in preterm infants born at or before 32 wk of gestation and to compare these values with those previously reported as occurring *in utero*; and second, to compare the relative amounts of Hb F and Hb A synthesis at a postconceptional age (gestational age + postnatal age) corresponding to term (38–42 wk) in the preterm-born infants with the values found in term-born infants studied at birth.

## METHODS

25 early preterm infants appropriate in weight for gestational age (2) with no evidence of congenital anomalies were selected for the study. They were carefully chosen at birth on the basis of a close correlation between the clinical estimate of gestational age, based on the one hand on external characteristics and neurological status, and on the other, on the menstrual history estimate. Their correlated gestational ages at birth ranged from 27 to 32 wk. Also included as term controls were 11 full-term infants who were appropriate in weight for gestational age (TAGA)<sup>1</sup> (2).

The proportional synthesis of Hb F and Hb A was determined by measuring the incorporation of [<sup>14</sup>C]leucine into the hemoglobins formed during the *in vitro* incubation of reticulocytes. The radioactive hemoglobins were prepared from samples obtained for analysis from the preterm infants immediately after birth and at 2–5-wk intervals, when clinical conditions permitted. Because of low reticulocyte yields, which resulted in little radioactive incorporation on one hand, and clinical conditions or mortality which prevented the withdrawal of blood on the other, only 7 of the infants had repeated studies performed, and of the 25 infants born at or before 32 wk of gestation, only 11 were available to be studied postnatally at their postconceptional age corresponding to term. The TAGA infants were sampled immediately after birth only. A total of 53 blood incubations were used in the study. The distribution of the birth weights and gestational ages of the infants studied is shown in Fig. 1.

The relative proportion of Hb F and Hb A synthesis was determined by methods described previously (1, 3). In brief, fresh heparinized reticulocyte-enriched blood samples (blood samples of 4–6 cm<sup>3</sup> were centrifuged at 4°C at 4,000 g for 10 min, the buffy coat was discarded and 1 cm<sup>3</sup> of packed cells from the top layer was used) were incubated under sterile

<sup>1</sup>Abbreviations used in this paper: CMC, carboxymethylcellulose; TAGA, term infant appropriate in weight for gestational age.

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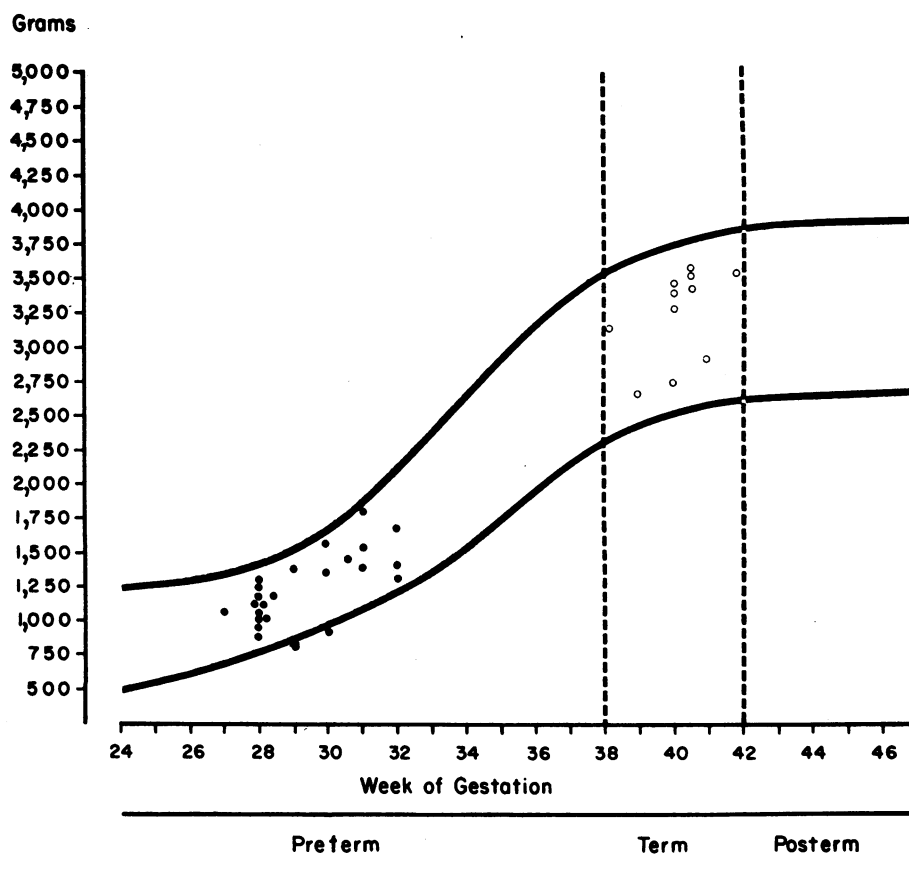


FIGURE 1 The distribution of the newborn infants on the Colorado intrauterine growth charts (4). ● are the preterm newborn infants, ○ are the term newborn infants.

conditions. To 2.64 cm<sup>3</sup> of an incubation mixture prepared by a modification (4) of the procedure devised by Borsook, Fischer, and Keighley (5), 1 cm<sup>3</sup> of packed erythrocytes 0.15 ml of ferrous ammonium sulfate (10.5 mg in 10 ml of 0.9% NaCl), and 0.1 ml [<sup>14</sup>C]leucine (L-[1-<sup>14</sup>C]leucine from Amersham/Searle Corp., Arlington Heights, Ill., specific activity 55.2 mCi/mmol) were added.

After a 6-h incubation carried out with agitation under air at 37°C, the cells were washed and lysed, and the hemolysate was desalted and purified by passage through a Sephadex G-25 column. The purified hemoglobin solution containing 60–80 mg hemoglobin was then subjected to column chromatography on a DEAE-Sephadex A-50 medium (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in the manner described by Huisman and Dozy (6), with columns 45 cm × 2.5 cm; the Sephadex height in the columns varied at the start of the elution between 18 and 25 cm. The hemoglobins were eluted at room temperature (20–21°C) with a decreasing pH gradient (7.9–7.1) of 0.5 M Tris-HCl at a flow rate of 20–26 ml/hr. Fractions of 4 ml were collected. The absorbance of the eluate was continuously measured at 280 nm.

Liquid scintillation-counting of the Hb A and Hb F fractions was carried out after preparation of the samples in the following manner. To 0.5 cm<sup>3</sup> of the eluted

fraction, 1.1 cm<sup>3</sup> of Bio-Solv-BBS-3 (Beckman Instruments, Inc., Fullerton, Calif.) as a solubilizer and 0.1 cm<sup>3</sup> of 50% hydrogen peroxide as a decoloring agent were added. The vials were then placed in a water bath at 50°C for 30 min. Finally 12 cm<sup>3</sup> of "cocktail" containing 4 g of pre-weighed Omnifluor (New England Nuclear, Boston, Mass.) in 1,000 cm<sup>3</sup> toluene was added to the vials. Counting was then performed in a Unilux 11 A liquid scintillation system (Nuclear-Chicago Corp., Des Plaines, Ill.). Counting efficiency varied between 77 and 81% on an external standardization quench curve. The external standard counts varied from +2.1% to -1.7% from the mean. The standard deviation of 20 repeated countings on the same sample was less than 1% of the mean. The percentage of radioactive Hb A and Hb F in duplicate blood samples after incubation and separation agreed within ±2%.

To demonstrate that the relative rate of Hb A and Hb F synthesis is constant throughout a 6-h incubation period, the following studies were carried out on blood samples from two newborn infants of different postconceptional ages. *a.* 1-cm<sup>3</sup> aliquots of reticulocyte-enriched packed erythrocytes obtained from cord blood from a 33-wk preterm infant were incubated for 30 min, 2 h, 5 h, and 6 h. The percentage of radioactive Hb F to total radioactive hemoglobin was determined for each of the incubations with the methods already

described in this paper. The radioactive fetal Hb as a percent of total radioactive Hb was 92.0% at 30 min, 86.5% at 2 h, 85.4% at 5 h, and 86.1% at 6 h. *b*. When a was repeated with cord blood from a term infant, born after 38 wk of gestation, the radioactive fetal Hb as a percent of total radioactive Hb was 70.6% at 2 h, 70.0% at 5 h, and 72.1% at 6 h. These studies demonstrated that after a sufficient incorporation of [<sup>14</sup>C]leucine, there is no significant change in the relative amounts of radioactive Hb A and Hb F throughout the incubation period used in this study.

In order to determine whether the minor Hb A<sub>1</sub> component in adult blood (7) (also known as Hb A<sub>1c</sub>) could account for some of the radioactivity attributed to Hb F, the following procedures were carried out.

A hemoglobin solution was prepared from 30 ml of fresh heparinized adult blood obtained from the hospital blood bank. Aliquots of 60–80 mg of adult hemoglobin were then subjected to DEAE-Sephadex chromatography (the methods used for preparing the hemoglobin solution and the DEAE-Sephadex chromatography were the same as those described above). The fractions that corresponded to Hb A<sub>1</sub> peak (7) were then pooled from the three chromatographic separations and concentrated by ultrafiltration.<sup>2</sup> Carboxymethylcellulose (CMC) column chromatography was then carried out on an aliquot of Hb A<sub>1</sub> alone. In addition, a mixture of radioactive Hb F derived from DEAE Sephadex separation and nonlabeled Hb A<sub>1</sub> was chromatographed in the same manner. These chromatograms were done using deionized 8 M urea buffers as described by Clegg, Naughton, and Weatherall (8). Before chromatography, the hemoglobin solutions were dialyzed against distilled water overnight, followed by 2 h of dialysis against the starting buffer. Finally, liquid scintillation counting was performed on 0.5 cm<sup>3</sup> aliquots of the fractions of gamma, beta (Hb A<sub>1</sub>), and alpha globin separated by the CMC column. There was no significant radioactivity detected in the beta (Hb A<sub>1</sub>) globin peak for the mixture of unlabeled Hb A<sub>1</sub> and radioactive Hb F. The optical density of the CMC chromatography and the counts per minute are shown in Fig. 2 (panels 1 and 4).

These findings demonstrate that the DEAE-Sephadex separations of Hb A and Hb F are complete when blood from newborn infants is used. The minor Hb A<sub>1</sub> component found in adult blood accounts for an insignificant amount of the radioactivity within the Hb F peak.

The completeness of the Hb A and Hb F separation was further substantiated by amino acid analysis of the Hb F obtained from a DEAE-Sephadex separation. The Hb F came from a preterm infant 4 wk of age and the analysis was performed with an automatic amino acid analyzer (Beckman model 121, Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.) (7). The ratio of isoleucine to leucine was 1:8.0 (the theoretical ratio in Hb F = 1:8.75). The ratio of isoleucine to phenylalanine was 1:3.58 (the theoretical ratio in Hb F = 1:3.75). The percentage of Hb F in Hb F + A<sub>1</sub> was 107% as calculated according to Schroeder, Huisman, Shelton, and Wilson (7) (error accepted = ±10%).

To compare the specific activities (sp act = cpm/OD<sub>280</sub>) of alpha globin in Hb F to alpha globin in Hb A the following study was carried out. A 20-cm<sup>3</sup> sample of fresh heparinized blood was withdrawn from a newborn infant undergoing an exchange transfusion. The reticulocyte count was 14%. The blood sample was centrifuged at 4,000 *g* at 4°C,

<sup>2</sup> Diaflo cell, Membrane UM-10, Amicon Corp., Lexington, Mass.

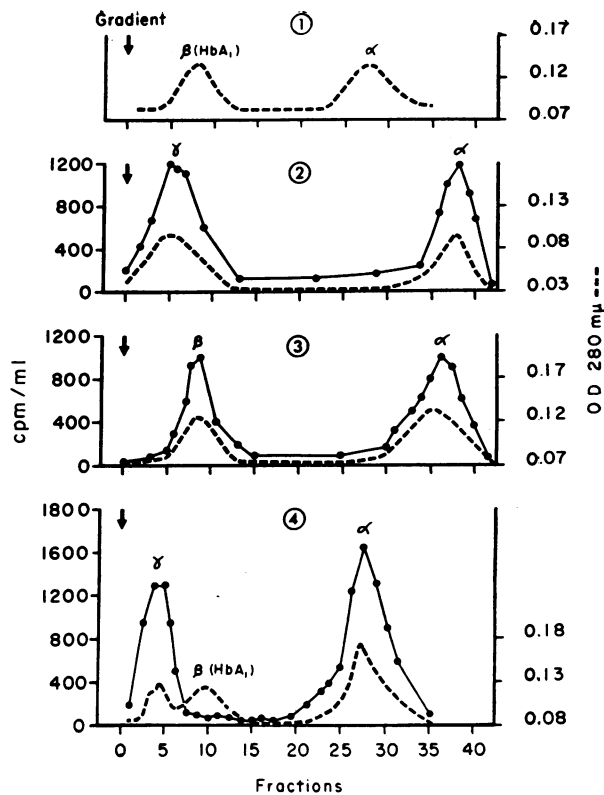


FIGURE 2 Gradient elution chromatography on CMC of globin from hemoglobins obtained after DEAE-Sephadex separation. Absorbance at 280 nm (---) and the counts (●—●—●) are superimposed: panel 1, Hb A<sub>1</sub>; panel 2, radioactive Hb F; panel 3, radioactive Hb A; panel 4, mixture of nonradioactive Hb A<sub>1</sub> and radioactive Hb F.

and 1 cm<sup>3</sup> of packed cells containing mainly immature erythrocytes was taken from the top layer and incubated. The hemoglobin solution prepared from this incubation was then subjected to DEAE-Sephadex column chromatography, and the eluted Hb A and Hb F fractions were each pooled and concentrated by ultrafiltration. CMC chromatography was performed on both the concentrated Hb F and Hb A solutions. Liquid scintillation counts were obtained from 0.5-cm<sup>3</sup> aliquots of the separated gamma, beta, and alpha globin fractions. The ratio of Hb A to Hb F alpha globin sp act was 1.09. The CMC chromatograms and counts per minute are shown in Fig. 2 (panels 2 and 3).

## RESULTS

Fig. 3 demonstrates the chromatograms and superimposed incorporation of [<sup>14</sup>C]leucine of the Hb A and Hb F fractions from a preterm newborn infant born at 32 wk of gestation whose hemoglobin synthesis was studied after 34, 36, and 39.5 wk of postconceptional age. The Hb A and Hb F peaks are symmetrical in shape, and the separations are complete. The proportion of Hb F

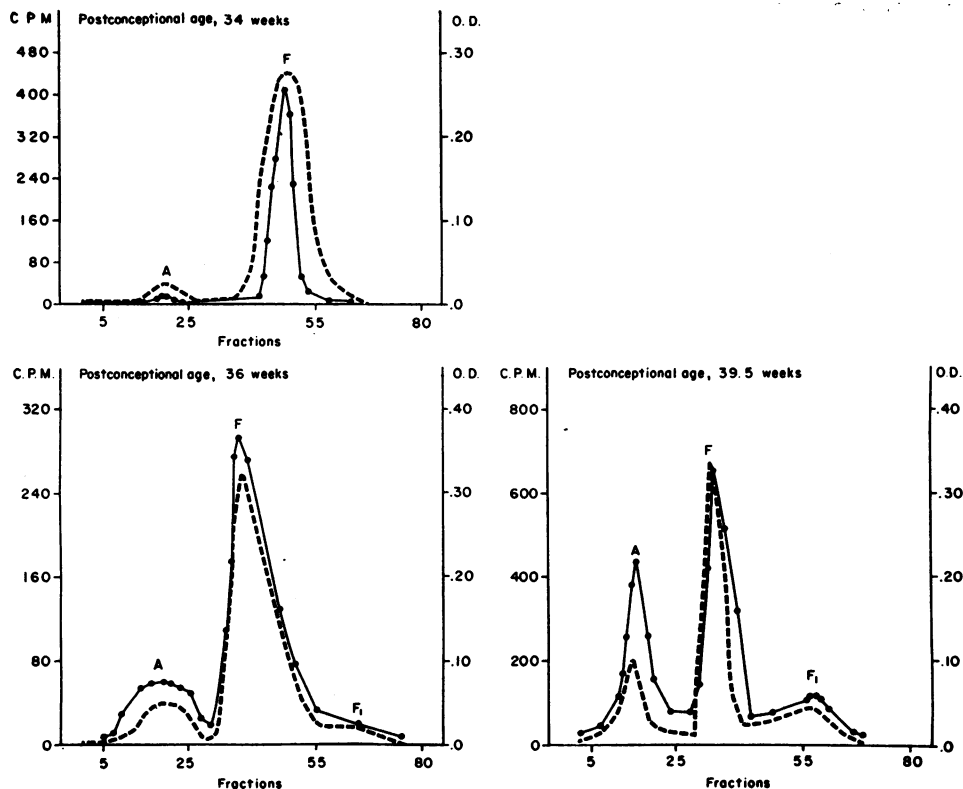


FIGURE 3 Postnatal A and F hemoglobin synthesis in an infant born at 32 wk of gestation, determined at the postconceptional ages of 34 wk (top), 36 wk (bottom left), and 39.5 wk (bottom right). The superimposed tracings represent the Sephadex-DEAE chromatographic separation of adult and fetal hemoglobins, absorbance 280 nm (---) and counts per minute of [ $^{14}\text{C}$ ]leucine-labeled hemoglobins (●-●-●). Top: total fetal hemoglobin 94.5%, radioactive fetal hemoglobin 95.1%. Bottom left: total fetal hemoglobin 88.3%, radioactive fetal hemoglobin 79.1%. Bottom right: total fetal hemoglobin 80.6%, radioactive fetal hemoglobin 66.8%.

being synthesized 2 wk postnatally was 95.1%, 1 mo postnatally, 79.1%, and at 7½ wk after birth, 66.8%.

Fig. 4 presents the data on the concentration of radioactive Hb F as a percentage of the total radioactive hemoglobin correlated with postconceptional age on the 25 preterm infants born at or before 32 wk of gestation. Also shown for comparison are the values of Hb F synthesis in the 11 term infants studied at birth. Confidence limits are also shown from a previous study representing *in utero* Hb F synthesis (1).

In all instances up to the postconceptional age of 37 wk, Hb F remained the major hemoglobin being synthesized. The birth process did not accelerate the transition towards Hb A synthesis.

Fig. 5 compares two distributions of radioactive Hb F as a percentage of total radioactive hemoglobin in relation to postconceptional age. On one hand, attention is first directed to the intrauterine values (dotted area) determined from cord blood at birth from newborn in-

fants of known gestational age, varying from 25 to 43 wk (1). This reflects intrauterine Hb A and Hb F synthesis. On the other hand, note the extrauterine values derived from the preterm infants born at or before 32 wk of gestation, studied postnatally. The differences between the two groups are unremarkable.

Fig. 6 illustrates the comparison of the synthesis of Hb F to total hemoglobin synthesis in two groups of infants both at the same postconceptional age of term; one group born at or before 32 wk of gestation and the other born at term. The means are 53.3%,  $\pm 15.7\%$  SD in the preterm group, and 59.7%,  $\pm 10.0\%$  SD in the term group. There is no statistically significant difference. ( $P < 0.2$ ).

## DISCUSSION

The separation of small amounts of Hb F in adult blood by chromatographic methods can be subject to error because of the minor component Hb A<sub>1</sub> (Hb A<sub>1c</sub>) related

to Hb A in the zone of Hb F (7). In the normal adult, Hb A<sub>1</sub> can amount to 5-8%. It has an amino acid composition identical to Hb A, with a chemical group the nature of which is not certain attached to the N terminus of a beta chain ( $\beta^{A_1}$ ) (9). Although it was not the purpose of this study to demonstrate chromatographic differences between  $\beta^{A_1}$ - and  $\beta$ -polypeptides, they appear to chromatograph as a single peak. In the neonatal period and especially when dealing with preterm infants where Hb F makes up the major portion of the total amount of hemoglobin, the quantity of Hb A<sub>1</sub> in the Hb F peak after DEAE-Sephadex separation is of no particular consequence.

Since incubations for this study were carried out in reticulocyte-enriched blood, the specific activity of the Hb A and Hb F are dependent upon the amount of unlabeled hemoglobins present. Because of the switch-over from Hb F to Hb A synthesis, the ratio of unlabeled Hb F to labeled Hb F will be greater than the ratio of unlabeled Hb A to labeled Hb A in the hemolysates used for chromatographic studies. Therefore, the specific activity of Hb A alpha globin would be greater than the specific activity of Hb F alpha globin when packed erythrocyte incubations from newborn infants are used to determine the relative proportions of Hb A and Hb F

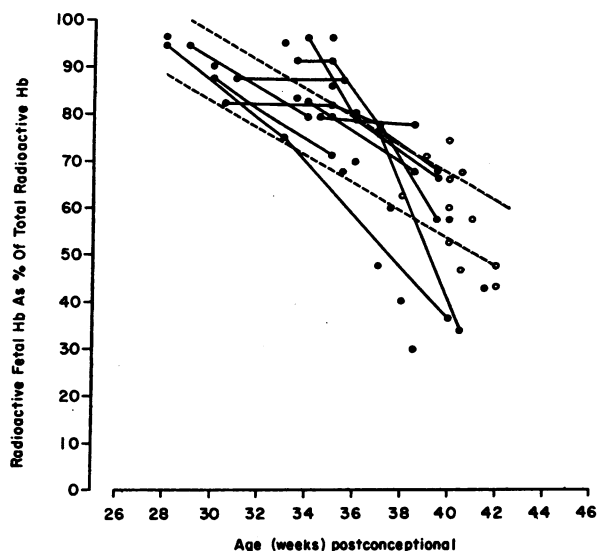


FIGURE 4 Radioactive fetal hemoglobin as a percentage of the total radioactive hemoglobin in relation to postconceptional age. ● are early preterm newborn infants, ○ are the term newborn infants, --- are the 95% confidence limits from a previous study reflecting *in utero* A and F hemoglobin synthesis (1). Lines uniting different points represent sampling of the same infant.

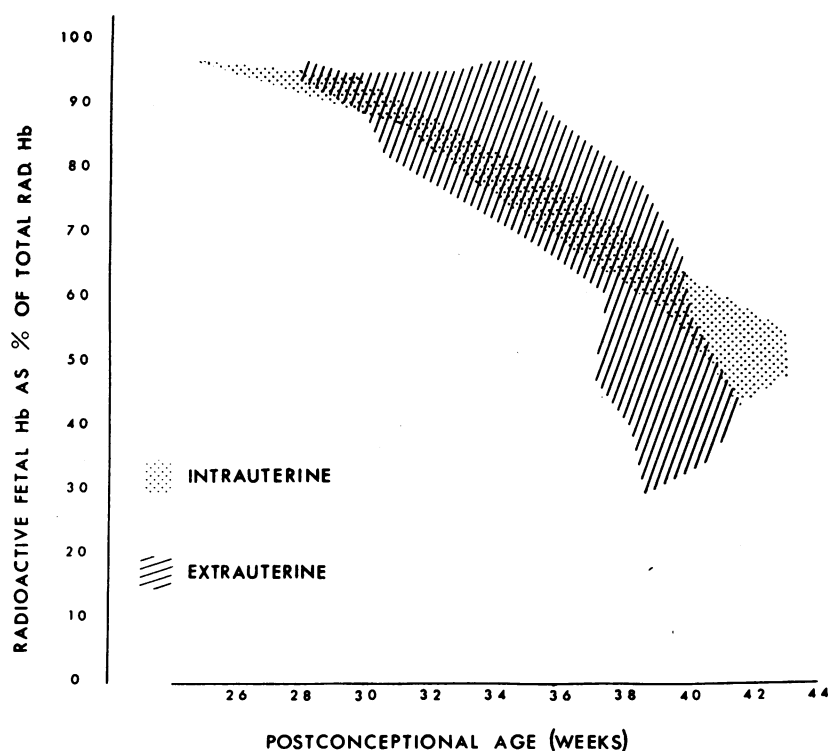


FIGURE 5 A comparison of the distribution of radioactive fetal hemoglobin as a percentage of total radioactive hemoglobin of infants of similar postconceptional age. Intrauterine group is described elsewhere (1).

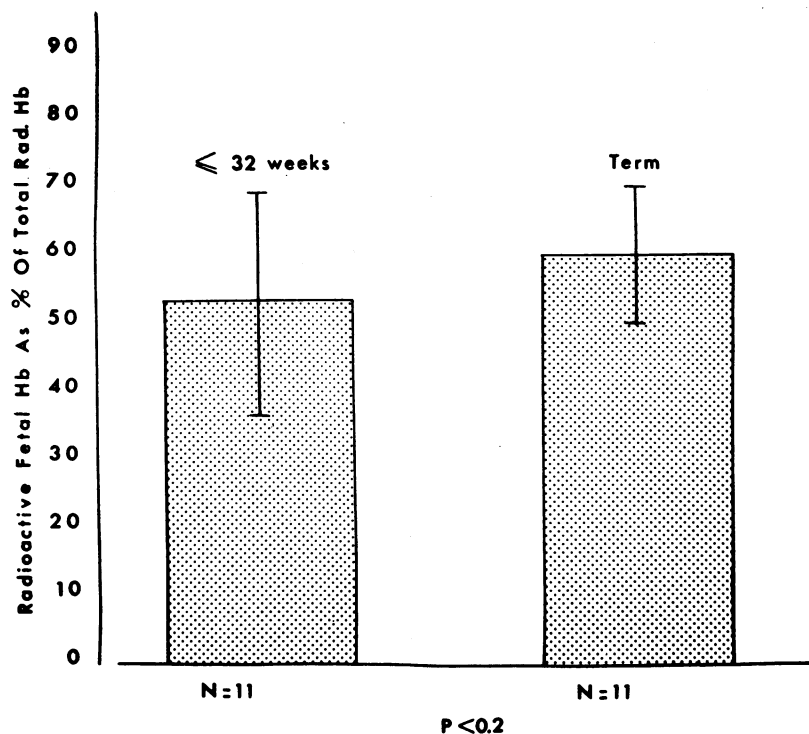


FIGURE 6 Radioactive fetal hemoglobin as a percentage of total radioactive fetal hemoglobin when determined at the postconceptional age corresponding to term in early preterm newborn infants, born at or before 32 wk of gestation ( $\leq 32$  wk), and determined at birth in infants born between 38 and 42 wk of gestation (term). Vertical bars refer to standard deviations.

synthesis. Any opportunity for an increase in Hb F was minimized by using a sample containing mainly immature erythrocytes. In this way, the ratio of Hb A to Hb F alpha globin approached 1.

The orderly appearance and disappearance of adult and fetal types of human hemoglobin as postconceptional age advances are perfect examples of the regulation and control of protein synthesis. They could provide an opportunity to study the genetic mechanisms involved and the influence of environmental factors.

In the normal early preterm newborn infant, there is a slow gradual decrease in the proportion of Hb F to total hemoglobin synthesis as postnatal age increases. Although a number of early preterm infants demonstrated a high level of Hb F synthesis during their first postnatal month, the transition does not differ remarkably from what was shown to occur *in utero*. There appears to be a slow transition towards Hb A synthesis which accelerates as the preterm infant approaches the 38th wk of postconceptional age, so that at the postconceptional age corresponding to term, there is no statistical difference between the early preterm-born group, which spent several months of extrauterine life, and the full-term newborn group.

The persistence of the high levels of Hb F synthesis postnatally in the early preterm newborn infants is clear indication that the transition from Hb F to Hb A synthesis is not influenced by the birth process. The rate of transition from Hb F to Hb A synthesis is species-specific, and the switchover is related to the rate of biological maturation (3), and is not affected in man by a precocious exposure to extrauterine life.

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