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# Hormonal Regulation of Hemoglobin Synthesis in Cells of Fetal Calf Liver Cultured in a Serum-Free Medium†

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ABSTRACT: A system of short-term cultures of fetal calf liver in a serum-free medium is described in which the effects of hormones and drugs on hemoglobin synthesis can be tested directly without interference from fetal calf serum normally used for this type of cell culture. In the model system described, hemoglobin synthesis was followed using the incorporation of $^{59}Fe$ into hemoglobin-associated heme or the incorporation of [$^3$H]leucine into fetal hemoglobins after their fractionation by acrylamide gel electrophoresis. Erythropoietin (0.2 μg/mL) stimulated $^{59}Fe$ incorporation into heme associated with hemoglobin in cell cultures isolated from fetuses of all the gestational ages studied, namely from 90 to 175 days. Testosterone ($10^{-8}$ M) was active only in cell cultures from fetuses of 115 to 155 days of gestation, while isoproterenol ($10^{-10}$ M) was active only in liver cells from fetuses of 115 to 135 days of gestation. Etiocholanolone ($10^{-8}$ M) had an effect similar to that of testosterone on hemoglobin synthesis but this was not significantly different from control values. At 115-180 days of gestation, the synthesis of adult hemoglobin in fetal liver cells is almost undetectable. There are two main fetal hemoglobins synthesized in vitro which have not been previously described. The first one ($F_0$) represents 50 to 70% of the fetal hemoglobin in vitro after a 5-h incubation with [$^3$H]leucine and is the main fetal hemoglobin of circulating red cells. $F_0$ is also the major hemoglobin synthesized by liver cell cultures after 16 to 24 h of incubation with [$^3$H]leucine. The fetal hemoglobin $F_1$ is only occasionally found in circulating red cells and when present it is less than 15% of the total hemoglobin. $F_1$ is found in the fetal liver in amounts of 15 to 50% of the total and is synthesized in large amounts in cell cultures in vitro using short incubation times with [$^3$H]leucine (5 h or less). Both testosterone and erythropoietin stimulated preferentially the synthesis of $F_0$. These results demonstrate that both hormones are not only stimulating total hemoglobin synthesis but also accelerating specifically the synthesis of the main fetal hemoglobin of mature red cells ($F_0$).

The human fetal liver is the main site of hemoglobin synthesis at midterm. The main hemoglobin being synthesized at this period of gestation is fetal hemoglobin ($\alpha_2\gamma_2$), although adult hemoglobin synthesis already represents 10% of the total hemoglobin synthesized (Kan et al., 1972; Hollenberg et al., 1972; Lanyon et al., 1975). It has been found that several hormones and drugs such as erythropoietin, testosterone, and isoproterenol stimulate the synthesis of hemoglobin or heme associated with hemoglobin in cell cultures of human fetal liver and that these hormones or drugs are active only at certain discrete periods of fetal development (Basch, 1972; Congote et al., 1974; Shchory and Weatherall, 1975; Congote and Solomon, 1977; Congote, 1977). Because the availability of human fetuses is very limited, further investigations in this field required the development of an experimental model with a pattern of fetal erythropoiesis similar to man. We chose the calf fetus as a model because it not only synthesizes fetal hemoglobin at midterm but also has the switch from fetal to adult hemoglobins similar to humans (Huisman, 1974; Kitchen and Brett, 1974). In this paper we describe the effects of a number of hormones on hemoglobin synthesis in fetal calf liver cells. We were able to test these hormones in liver cells cultured in a serum-free medium, thus excluding any interaction with fetal calf serum which was a drawback of the media previously employed in such studies.

## Materials and Methods

*Materials.* All materials for tissue culture were purchased from Flow Laboratories. Bovine serum albumin (fraction V) and DL-isoproterenol were purchased from Sigma, and bovine transferrin was from Miles or Calbiochem. Sheep plasma erythropoietin (step III) was purchased from Connaught Laboratories, Toronto, Canada. The erythropoietin preparations used had specific activities of 2.2, 4.5, and 5 units/mg. $^{59}FeCl_3$ (specific activities 10-25 mCi/mg of Fe) was obtained from Frosst-Merck Laboratories, Montreal, Canada. L-[4,5-$^3$H]Leucine (53-60 Ci/mmol) and L-[U-$^{14}$C]leucine (330

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mCi/mmol) were purchased from Amersham-Searle and Minicon A25 cells from Amicon.

*Preparation of Cell Cultures.* Cells were prepared from livers obtained from calf fetuses collected at a local abattoir. The livers were washed, chopped, and trypsinized as previously described (Congote et al., 1974). A single trypsinization step of 15 min was sufficient to obtain a large enough number of cells for the experiments to be described. The cells were suspended in F-12 medium prepared in Hanks balanced salt solution with 1.5 g/L additional $NaHCO_3$, double amounts of amino acids and sodium pyruvate, 50 units/mL each of penicillin and streptomycin, and supplemented with 300 $\mu$g/mL bovine serum albumin and 30 $\mu$g/mL bovine transferrin. In experiments where $^{59}Fe$ incorporation was determined, the transferrin was added as [$^{59}Fe$]transferrin prepared as indicated below. The medium did not contain any $FeSO_4$. A total of $30 \times 10^6$ cells were plated in 3.5-cm tissue-culture dishes (Falcon) containing 1 mL of the F-12 medium with or without hormones. The hormones were added directly to the medium and sterilized by filtration through nitrocellulose filters. No loss of erythropoietic activity was observed if the hormones were mixed with albumin before filtration. In experiments where [$^3H$]leucine incorporation was studied, dishes of 6-cm diameter (Falcon) were used containing 3 mL of medium and $90 \times 10^6$ cells. All incubations were performed in a $CO_2$ incubator at 37 °C with 5% $CO_2$ in humidified air. For the determination of hemoglobin synthesis in peripheral blood, heparinized blood was taken by heart puncture and centrifuged. The buffy coat was removed and the cells were washed three times with Hank's balanced salt solution. The cells were suspended in 3 mL of the F-12 medium as indicated above and incubated in 6-cm tissue-culture dishes using a cell density of $10^8$ cells/dish.

*$^{59}Fe$ Incorporation and Heme Synthesis.* $^{59}Fe$-labeled transferrin was prepared with $^{59}FeCl_3$ and iron-free transferrin as described by Martinez-Medellin and Shulman (1972). The final concentration of $^{59}Fe$ and transferrin in the medium was 2 $\mu$Ci/mL and 30 $\mu$g/mL, respectively. The method used for the extraction of heme has been previously described (Congote et al., 1974).

*Analysis of the Hemoglobins.* Analysis of fetal and adult hemoglobins was performed using electrophoresis on cellulose acetate (Seprapore III, Gelman) according to the procedure described by the manufacturer. The hemoglobins on the cellulose strips were stained with Ponceau S and scanned using a Gelman densitometer. Acrylamide gel electrophoresis was performed as previously described (Congote et al., 1974). The quantitation of unlabeled hemoglobins was done by the method of Drabkin (1945), after elution of the bands in Drabkins' solution. Labeled hemoglobins were analyzed as previously described (Congote et al., 1974). The chromatography of hemoglobins on CM-cellulose was done by a slight modification of the method of Schroeder et al. (1976). The columns were prepared and equilibrated in 15 mM Bistris, pH 6.1, containing 0.1 g/L KCN. Fetal and adult hemoglobins were eluted with a gradient of 375 mL of 5 mM NaCl and 375 mL of 80 mM NaCl prepared in the same Bistris buffer indicated above. Samples of 5 mL were collected and nonlabeled fetal and adult hemoglobins were added prior to chromatography to localize the fractions from the gradient containing these hemoglobins. Aliquots of 2 mL were mixed with 10 mL Insta-Gel (Packard) and counted using a Packard TriCarb liquid scintillation counter, Models 4322 and 3950.

## Results

Liver cells from calf fetuses were prepared using the method previously described by us for the preparation of human fetal liver cells (Congote et al., 1974). After trypsinization and centrifugation, the supernatant of fetal calf livers was turbid, indicating cell destruction. The pellets were mostly red, surrounded by a yellow ring which could easily be removed with a Pasteur pipet. Microscope smears of trypsinized cells prepared with Wrights' stain indicated that the cells were erythroid (mostly basophilic) and that the amount of parenchymal cells was very low (about 1%). Thus, the method of trypsinization seemed to be suitable for the preparation of an enriched fraction of calf liver erythroid cells. To confirm this view, the hemoglobin content of fetal calf red cells, liver homogenates, and trypsinized liver cells was determined over a wide span of gestation as shown in Figure 1. The amount of hemoglobin present per gram wet weight was higher in cells prepared from older fetuses, indicating that a larger proportion of mature red cells was present in the preparations from fetuses at advanced stages of gestation. The hemoglobin content of the trypsinized cells was much lower than that found in the mature circulating fetal red cells (Figure 1), indicating that the cells obtained after trypsinization are at a relatively early stage of erythroid cell differentiation (Denton et al., 1975).

We then measured hemoglobin synthesis in the fetal calf cell cultures by two methods in order to determine whether the

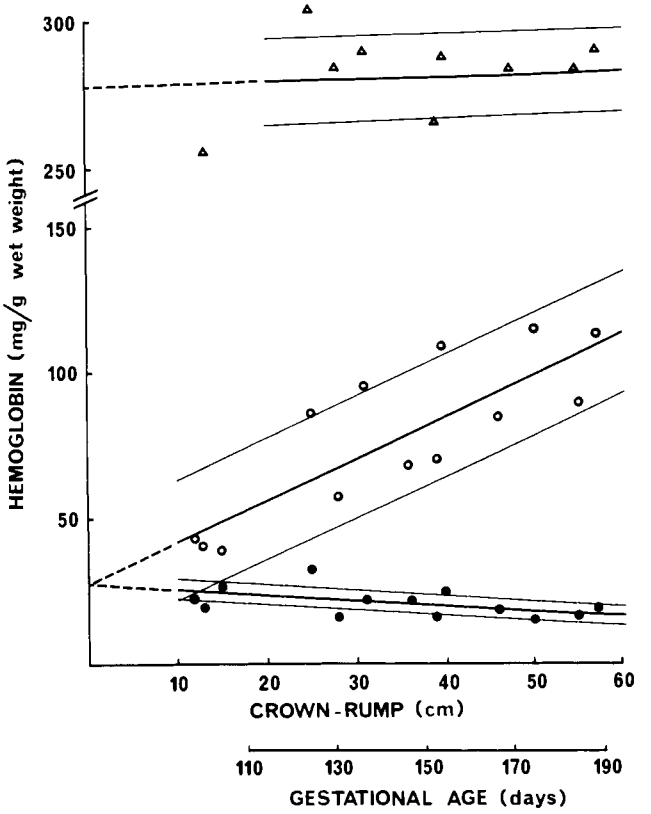


FIGURE 1: Hemoglobin content of fetal red cells, liver homogenates, and trypsinized liver cells. Heparinized blood was taken from calf fetuses of different gestational ages by heart puncture. The livers were removed and washed, an aliquot was used for the preparation of liver cells as described under Methods, and a second portion was used for the preparation of liver homogenates. After washing three times with Hank's balanced salt solution, the cells were lysed in Drabkin's solution and centrifuged at 12 000 $\times g$ for 15 to 20 min to remove cell debris, and hemoglobin was measured by the method of Drabkin (1945). Liver homogenates were prepared in Drabkin's solution using a Teflon glass homogenizer until complete cell disruption was obtained. The cell debris, nuclei, and mitochondria were removed by centrifugation. Closed circles are the hemoglobin content of liver homogenates, open circles represent hemoglobin in trypsinized liver cells, and triangles are the hemoglobin content of peripheral red blood cells. Regression lines ± confidence limits for $P < 0.01$.

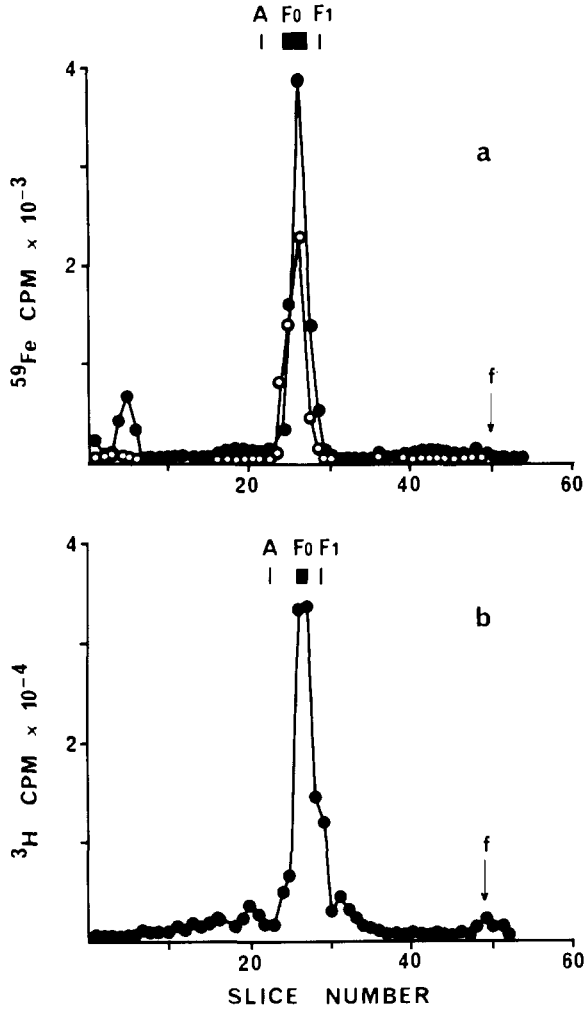


FIGURE 2: Polyacrylamide gel electrophoresis of the $^{59}$Fe-containing proteins of the supernatants from cell lysates. The cells were incubated for 18 h in the presence of 0.2 units/mL of erythropoietin: (a) $^{59}$Fe incorporation; (b) [$^{3}$H]leucine incorporation. In the case of $^{59}$Fe incorporation, the cells from four dishes were pooled, lysed, and analyzed on two parallel acrylamide gels. Closed circles in a designate total $^{59}$Fe incorporation; the open circles in a represent total heme. The lines A, $F_0$, and $F_1$ indicate the positions of adult hemoglobin, the main fetal hemoglobin, and the minor fetal hemoglobin, respectively. The f marks the front of the electrophoretic run, as indicated by the position of bromophenol blue. The position of adult hemoglobin was localized by adding nonlabeled carrier, whereas $F_0$ and $F_1$ were the natural hemoglobins present in the cell lysate.

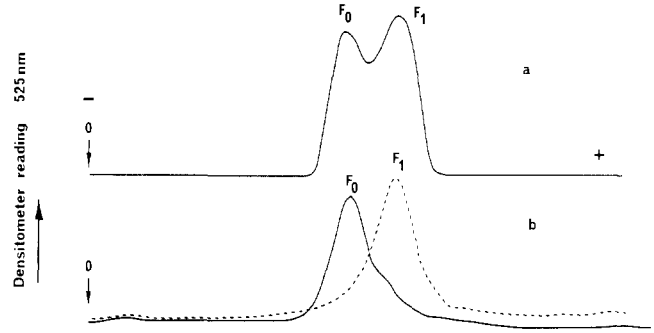


FIGURE 3: Densitometer trace of fetal hemoglobins following cellulose acetate electrophoresis. Three samples were spotted simultaneously on a cellulose acetate strip: (a) the densitometry scan from a liver cell lysate with almost equal amounts of $F_0$ and $F_1$; and (b) the scan of hemoglobins $F_0$ and $F_1$ previously eluted from acrylamide gel. The solid line represents hemoglobin $F_0$, mobility 0.57; the dotted line, hemoglobin $F_1$ with a mobility 0.63. The cellulose strip was fixed in trichloroacetic acid, stained with Ponceau S, and analyzed in a Gelman densitometer.

procedure previously established for human fetal liver (Congote et al., 1974) was applicable to the present studies. The first method consisted of $^{59}$Fe incorporation into iron-containing soluble cell proteins followed by extraction with butanone which extracts the $^{59}$Fe incorporated into heme. In order to have a sufficient amount of radioactivity which would allow us to identify the nature of the $^{59}$Fe-labeled soluble ferroprotein separated on acrylamide gels, the following procedure was adopted. The cells from four dishes were pooled following incubation in the presence of erythropoietin (Goldwasser et al., 1975) and a long incubation time of 18 h was used in the presence of [$^{59}$Fe]transferrin as shown in Figure 2a. Even with this very long incubation time, the $^{59}$Fe incorporated into hemoproteins corresponded in mobility to hemoglobin, showing the specificity of this method of labeling for measuring $^{59}$Fe incorporation into hemoglobin. It is surprising that cells cultured under identical conditions using [$^{3}$H]leucine as precursor showed that the incorporation into hemoglobin was highly specific (Figure 2b). We utilized the $^{59}$Fe incorporation to determine the optimal conditions for the hormonal stimulation of total hemoglobin synthesis in the cells. This allowed us to screen a large number of cell cultures and was also the same procedure previously employed to study hormonal stimulation of heme synthesis in human fetal liver cells (Congote et al., 1974; Congote and Solomon, 1977). Table I shows the data obtained following the stimulation of hemoglobin synthesis by erythropoietin, isoproterenol, and testosterone. The cells were incubated with the hormones immediately after trypsinization. Preliminary experiments had indicated that the hormones had to be added shortly after preparation of the cells to observe an effect. With human fetal liver cells the hormones can be added 1 day after trypsinization (Congote et al., 1974). Of the three erythropoietic factors, erythropoietin seemed to have the highest stimulatory effect at all gestational ages studied. Testerosterone had a lower but significant effect on the synthesis of hemoglobin. This may be of physiological importance because the red cell count in fetuses between 112 to 210 days of gestation is generally higher in males ($5.2 \times 10^6 \pm 0.3 \times 10^6$ cells/$\mu$L) than in females ($4.6 \times 10^6 \pm 0.3 \times 10^6$ cells/$\mu$L). The numbers are means $\pm$ standard error of the mean for ten determinations.

Having determined the period in gestation where both erythropoietin and testosterone (115–155 days) actively stimulate hemoglobin synthesis, we analyzed in greater detail the nature of the adult and fetal hemoglobins synthesized. For these studies we labeled the hemoglobins with [$^{3}$H]leucine. In preliminary studies we noticed that the calf fetal liver contained variable amounts of a second hemoglobin designated $F_1$ which had a different mobility on electrophoresis from the main fetal hemoglobin of circulating red cells designated here as $F_0$. The electrophoretic mobility in acrylamide gels for the main fetal hemoglobin, $F_0$, was 0.57 (using bromophenol blue as the standard), whereas $F_1$ had a mobility of 0.63. Both $F_1$ and $F_0$ could be readily separated from adult hemoglobin on acrylamide gels (mobility = 0.49). The bands of $F_0$ and $F_1$ could be easily observed in acrylamide gels without staining and it was possible to cut them out and elute them from the gels. Figure 3a shows the separations of $F_0$ and $F_1$ on cellulose acetate electrophoresis of a liver cell lysate containing approximately the same amount of both hemoglobins. When the $F_0$ and $F_1$ bands separated by acrylamide gel electrophoresis are eluted from the gels and are concentrated using "minicon A25" cells and then applied on cellulose acetate strips, a good separation of both hemoglobins is obtained after electrophoresis (Figure 3b). The same method can be used for the separation of labeled

TABLE I: Effects of Erythropoietin, Isoproterenol, Testosterone, and Etiocholanolone on the $^{59}Fe$ Incorporation into Heme Associated with Hemoglobin in Cell Cultures of Fetal Calf Liver.[a]

| Gestational age (days) | $^{59}Fe$ incorp into heme (dpm/dish) | Ratios of treated cells/controls | | | |
|---|---|---|---|---|---|
| | | Erythropoietin | Isoproterenol | Testosterone | Etiocholanolone |
| 90-115 | 4200 ± 600 | 1.90 ± 0.60 | 0.91 ± 0.23 | 0.96 ± 0.17[a]<br>0.92 ± 0.13[b]<br>0.90 ± 0.13[c] | 0.87 ± 0.14 |
| 115-135 | 1600 ± 200 | 1.54 ± 0.18 | 1.54 ± 0.28 | 1.18 ± 0.20[a]<br>1.34 ± 0.28[b]<br>1.39 ± 0.30[c] | 1.12 ± 0.18 |
| 135-155 | 1700 ± 300 | 1.85 ± 0.16 | 0.90 ± 0.19 | 1.26 ± 0.18[a]<br>1.34 ± 0.16[b]<br>1.23 ± 0.12[c] | 1.14 ± 0.27 |
| 155-175 | 1000 ± 100 | 1.76 ± 0.35 | 1.00 ± 0.05 | 1.06 ± 0.05[a]<br>1.11 ± 0.16[b]<br>0.78 ± 0.10[c] | 1.11 ± 0.12 |

[a] The cells were incubated for 18 h with the hormones and then for 5 h with [$^{59}Fe$]transferrin. After incubation the cells were washed and lysed and the $^{59}Fe$ incorporated into heme associated with hemoglobin was analyzed as indicated in Methods. The concentrations used were: erythropoietin, 0.2 units/mL; DL-isoproterenol, $10^{-10}$ M; testosterone, (a) $10^{-10}$ M, (b) $10^{-8}$ M, and (c) $10^{-6}$ M; etiocholanolone, $10^{-8}$ M. The number of fetuses used were: seven of 90-115 days gestation, nine of 115-135 days gestation, seven of 135-155 days gestation, and five of 155-175 days gestation. The values shown are the mean ± standard error of the mean. The differences between hormone-treated cells and controls were tested using Fisher's paired $t$ test. These differences were significant for erythropoietin in cultures from fetuses of 90-175 days ($P < 0.002$), for isoproterenol in cultures from fetuses of 115-135 days ($P < 0.05$), and for testosterone ($10^{-8}$ M) in cultures of 115-155 days ($P < 0.05$). The effects of etiocholanolone were not significant.

$F_0$ and $F_1$ but not for the separation of adult hemoglobin from labeled $F_0$. The synthesis of adult hemoglobin in cultures of fetal calf liver cells is very low. For this reason, all the radioactivity associated with the adult hemoglobin band in acrylamide gels turned out to be a contamination of $F_0$. This was observed by repeated electrophoresis of the adult hemoglobin band (results not shown). As a result, the amounts of adult hemoglobin synthesized were evaluated after chromatography on carboxymethylcellulose, a system which gives a good separation of fetal and adult hemoglobins (Figure 4). In Figure 4, $F_1$ and $F_0$ are eluted together from the column. We could not observe any significant amounts of adult hemoglobin synthesis in calf liver cells isolated from fetuses of 100 to 180 days of gestation. This is a major difference from the human fetal liver cells, where adult hemoglobin represents about 7 to 11% of the total hemoglobin synthesized (Kan et al., 1972; Hollenberg et al., 1972). In subsequent studies using the procedure of hemoglobin synthesis described by Alter et al. (1976), we noted an additional peak which was eluted from the CM-cellulose column much later than adult hemoglobin. This peak represented approximately 3% of the total $^3H$ incorporated into hemoglobin.

The two main hemoglobins present in the liver, $F_0$ and $F_1$, were compared to the hemoglobins present in peripheral blood cells. The hemoglobins were separated by acrylamide gel electrophoresis as shown in Figure 3. Hemoglobin was measured by the method of Drabkin after elution of the bands and pooling material from eight gels. The average $F_0/F_1$ ratio from trypsinized liver cells was 2.8 with values ranging between 1 and 4.5, whereas peripheral blood cells had only a very small amount of $F_1$. In fact, the lowest $F_0/F_1$ ratio measured in these cells was 6. We also measured the synthesis of both hemoglobins after a 5-h incubation with [$^3H$]leucine. Here again the $F_0/F_1$ ratios from the in vitro synthesized hemoglobins were higher in peripheral red cells (average 1.8, with values between 1.4 and 2) than in liver cells (1.1, values between 0.5 and 1.8). The difference in the $F_0/F_1$ ratios observed after the 5-h incubation with [$^3H$]leucine in liver cells and peripheral red cells

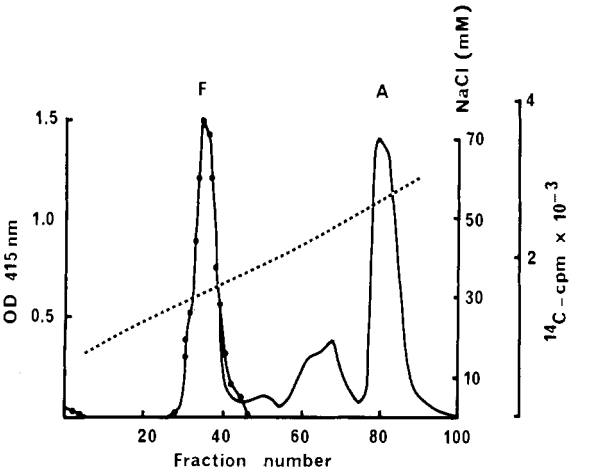


FIGURE 4: CM-cellulose chromatography of hemoglobins synthesized in vitro in fetal calf liver cells. A typical chromatographic run is shown in this figure. Liver cells from a fetus of 105 days gestation were incubated for 24 h with [$^{14}C$]leucine, washed, and lysed. The supernatant from the lysate was mixed with nonlabeled fetal and adult hemoglobins and the mixture was dialyzed overnight against 15 mM Bistris, pH 6.1, containing 0.01% KCN. The proteins were applied to a 0.9 × 20 cm carboxymethylcellulose column and eluted as indicated under Methods. The solid line represents optical density at 415 nm and the dotted line represents the concentration of NaCl. Closed circles indicate [$^{14}C$]leucine incorporation. The small peaks between the two major fractions F (fetal) and A (adult) correspond to minor hemoglobins present in the adult hemoglobin sample used as a carrier.

was significant ($p < 0.02$, Fisher unpaired $t$ test). Because the trypsinized liver cells contain a high proportion of immature erythroid cells, $F_1$ seems also to be more abundant in immature red cells and its synthesis is high in the tissue-culture system employed here. This is particularly striking when short-term incubations are performed, 5 h or less (Figure 5), whereas after a long incubation with [$^3H$]leucine the main hemoglobin being synthesized is $F_0$.

The role of erythropoietin and testosterone in the synthesis

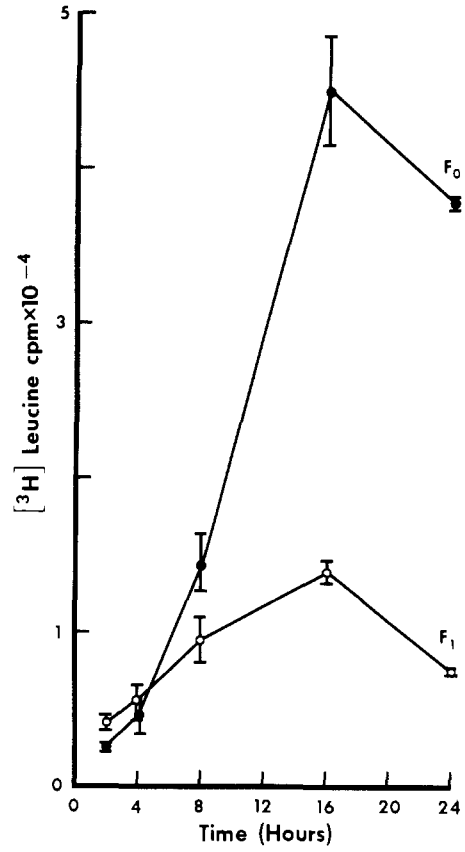


FIGURE 5: [$^3$H]Leucine incorporation into $F_0$ and $F_1$ hemoglobins as a function of time of incubation. The isolated fetal liver cells were incubated with [$^3$H]leucine for the time periods indicated on the abscissa. At the end of the incubation, the cells were washed and lysed and $F_0$ and $F_1$ were separated on acrylamide gels as indicated in the text. Open circles represent [$^3$H]leucine incorporated into $F_1$ and closed circles represent [$^3$H]leucine incorporated into $F_0$. The values are averages of two determinations using cells of the same fetus. Although the ratios $F_0/F_1$ may vary from one fetus to the other, the synthesis of $F_0$ was significantly higher in long-term incubations (16–24 h) than in short-term incubations (2–4 h) ($P < 0.01$, Fisher's unpaired $t$ test).

TABLE II: Effects of Testosterone and Erythropoietin on the Synthesis of $F_0$ and $F_1$ by Calf Fetal Liver Cells.[a]

| Hormone treatment | $F_0$ | $F_1$ |
|---|---|---|
| Controls | 32 000 ± 4200 dpm | 32 000 ± 3500 dpm |
| | Hormone-treated cells/controls | |
| Testosterone, 10 nM | 1.31 ± 0.07 $P < 0.02$ | 0.86 ± 0.17 |
| Erythropoietin, 5 milliunits/mL | 1.21 ± 0.09 | 1.00 ± 0.08 |
| Erythropoietin, 0.2 units/mL | 1.67 ± 0.24 $P < 0.05$ | 1.33 ± 0.26 |

[a] Liver cells were cultured with the hormones for 18 h and then for 5 additional hours with [$^3$H]leucine (50 $\mu$Ci/3 mL of incubation medium). The cells were washed and lysed as indicated in Methods. The hemoglobins were separated by acrylamide gel electrophoresis and counted. The numbers represent the average of five experiments ± standard error of the mean.

of these hemoglobins was investigated and the results are shown in Table II. The incorporation of [$^3$H]leucine into $F_0$ and $F_1$ was practically the same for both hemoglobins in the control experiments. Both testosterone and erythropoietin stimulated preferentially the synthesis of $F_0$, the main hemoglobin of circulating red cells. Erythropoietin could also stimulate $F_1$ at high concentrations but the stimulation of $F_0$ was even greater at this concentration.

## Discussion

The model system of fetal calf liver in the cell culture described here is a good alternative for the human fetal liver system previously described (Congote et al., 1974). The advantages of this system for the study of hormonal regulation of liver erythropoiesis are that it has fetal hemoglobins and that it can be studied throughout gestation. Furthermore, the effects of hormones can be studied in a serum-free medium containing only small amounts of albumin and transferrin. The experiments can be done with a purified erythroid cell fraction, because of the rapid destruction of many hepatocytes during trypsinization (Figure 1). This has enabled us to study the effects of hormones such as erythropoietin and testosterone as well as isoproterenol on fetal hemoglobin synthesis in short-term cultures of liver cells over a wide range of gestation (Table I).

Testosterone has been shown to stimulate $^{59}$Fe incorporation into hemoglobin only in liver cells obtained between 115 and 155 days of gestation, in contrast to isoproterenol which has an effect only between 115 and 135 days (Table I). This phenomenon is very similar to the effects of testosterone and isoproterenol observed in human fetal liver cells (Congote et al., 1974; Congote and Solomon, 1977; Congote, 1977), where isoproterenol was shown to have its optimal activity at 8–10 weeks of gestation and testosterone had peak activity at 10–13 weeks. In calf liver cells (Table I) there is a partial overlap in the time of gestation for optimal action of testosterone and isoproterenol. Etiocholanolone, a 5$\beta$-H reduced steroid, has a similar effect to that of testosterone on hemoglobin synthesis, but it was not significantly different from control values. Testosterone was not metabolized during the short incubation period used in these experiments (S. Mulay, unpublished results). This is probably due to the small number of hepatocytes present in the cultures. There is a major difference between human and calf liver cells in relation to their response to erythropoietin. There is a definite gestational age-dependent stimulation of hemoglobin synthesis due to erythropoietin in cell cultures of human fetal livers (Basch, 1972), whereas in fetal calf liver cells the hormone stimulates hemoglobin synthesis in cells from fetuses of all the gestational ages studied (Table I). There is apparently no appearance or disappearance of target cells for erythropoietin between 90 and 175 days of gestation. The small decline of erythropoietin-mediated hemoglobin synthesis at 115–135 days of gestation was not significantly different from the effects of the hormone at other gestational periods. Another major difference between human and calf fetal liver cells is that there is practically no detectable synthesis of adult hemoglobin in calf cells (Figure 4).

The two major human fetal hemoglobins are difficult to separate because the $\gamma$-chain structures are almost identical (Schroeder and Huisman, 1974). The cow has at least two main fetal hemoglobins which can be readily separated by electrophoresis. We have named these hemoglobins $F_0$ and $F_1$. $F_0$ is by far the major hemoglobin in circulating fetal red cells. It is interesting to note that the amount of the minor fetal hemoglobin, $F_1$, changes considerably from one fetus to the other and is much higher in fetal liver cells than in peripheral red cells, indicating its preferential localization in immature erythroid cells. For this reason, $F_1$ can be classified as a transient hemoglobin present in immature red cells but not in mature erythrocytes, as has been described for the rabbit by Borsook (1968). Our in vitro culture system seems to be par-

ticularly appropriate for the synthesis of $F_1$ in short-term incubations with [$^3$H]leucine (Figure 5). In a few instances $F_1$ is synthesized in larger amounts than $F_0$. Peripheral red cells synthesize more $F_0$ than $F_1$, again showing some kind of preferential switch from $F_1$ to $F_0$ during erythroid cell maturation. The biochemical characteristics of $F_1$ and $F_0$ are now under investigation. It remains to be seen if the differential synthesis of both hemoglobins is due to different transcription rates or due to a preferential degradation of $F_1$ (or the mRNA for the globin chains of $F_1$) during erythroid cell differentiation. Clissoid et al. (1977) have found that a $\beta$-related globin mRNA in rabbit proerythroblasts and basophilic erythroblasts represents 50% of the total globin mRNA and is absent in reticulocytes. This globin mRNA probably corresponds to the minor hemoglobin typical of immature erythroid cells (Borsook, 1968). This could be operative for $F_1$ in the calf fetus.

An alternative mechanism of regulation could take place at a posttranscriptional level. There are three main possibilities of control: RNA processing and translation and posttranslational modification of hemoglobins. Some posttranslational modifications of hemoglobins, such as acetylation or glycosylation, could account for the different electrophoretic mobilities of $F_0$ and $F_1$ (Basch, 1972; Koenig et al., 1977). These possible mechanisms have not as yet been investigated in our system.

A preferential switch for $F_1$ to $F_0$ has also been observed after incubation with erythropoietin and testosterone (Table II). This means that these two hormones not only stimulate in a general way red cell differentiation but also specifically increase the main fetal hemoglobin of mature red cells ($F_0$). It would be of interest to determine whether these hormones are involved in the switch mechanisms of $^G\gamma$ and $^A\gamma$ in human fetal liver. If this is the case, the calf fetus could be a model for the study of phenomena of transitions of hemoglobins during fetal development or unusual $^G\gamma/^A\gamma$ ratios.

In conclusion, we have found a direct stimulatory effect of erythropoietin, testosterone, and isoproterenol on hemoglobin synthesis in cultures of fetal calf liver cells. With the exception of erythropoietin, which was active in cells of fetuses of all gestational ages studied, the optimal effects of testosterone and isoproterenol showed a similar dependence on gestational age as was found for human fetal liver cells. Erythropoietin and testosterone not only stimulate hemoglobin synthesis but have also a qualitative effect, in that both hormones specifically increase the amount of $F_0$, the main hemoglobin of circulating fetal calf red cells.

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