Hormone Control of Heme Synthesis in Cultures of Human Fetal Liver Cells†

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ABSTRACT: Heme synthesis and its regulation by several hormones have been studied in cell cultures from livers of human midterm fetuses. Freshly prepared primary cultures have a very active synthesis of heme. The electrophoretic analysis of the water-soluble heme proteins shows that at this stage of culture the newly synthesized heme is almost exclusively part of hemoglobin. Heme synthesis associated with this water-soluble cell fraction declines rapidly in older cultures and can be observed again only after subculture, where most of the heme is in a free dialyzable form and not as part of hemoglobin. In both freshly prepared primary cultures and subcultured fibroblast-like cells, the synthesis of heme can be stimulated by testosterone, 5α- and 5β-androstanediol, and dibutyryl-cAMP. The enhancement of heme synthesis is accompanied by a less pronounced but significant stimulation of iron incorporation into other cell components. Among the steroids tested, testosterone was the most active in primary cultures, whereas 5β-androstanediol was most effective in subcultures. The steroid-mediated enhancement of heme synthesis can be blocked by α-amanitin, actinomycin D, cycloheximide, and ethidium bromide, suggesting the involvement of *de novo* RNA and protein synthesis in this process. The sensitivity of the primary cells toward testosterone varies with the gestational age of the fetus. The hormone can stimulate heme synthesis in cells from fetuses of 10–13 weeks gestation, whereas cells from fetuses of 9–10 weeks or 13–16 weeks do not respond or respond very weakly to testosterone stimulation. Testosterone is rapidly metabolized in primary cultures to androstenedione and etiocholanolone, whereas in subcultured cells the metabolism is slow and no etiocholanolone could be found. Two other important differences of the two systems are the incubation time with the steroid necessary to see a stimulation of heme synthesis, which was 6 hr in primary cultures and 48 hr in subcultures. In addition, human erythropoietin was a potent stimulator of heme synthesis in primary cultures but not in subcultured cells.

Heme is an essential component of hemoglobin, cytochromes, and many enzymes and its rate of synthesis affects a considerable number of cell processes. Although the biosynthesis of heme is the result of a complex chain of reactions, it is regulated by a rate-limiting enzyme, δ-aminolevulinic acid synthetase (Granick, 1966). Heme synthesis during embryonic development has been studied in a number of systems in relation to the formation of red cells (Cole *et al.*, 1968; Marks, 1972; Granick and Kappas, 1967). There are many similarities in the pattern of red cell formation during ontogenesis of various vertebrates (Ingram, 1972). Due to the variations in the endocrine regulatory systems of different species, it is of particular importance to use human cells for the study of hormonal regulation of fetal erythropoiesis in man. Among the different factors that may act as physiologic inducers of erythropoiesis in the fetus, erythropoietin has been the most widely studied, particularly in mice (Cole *et al.*, 1968; Marks, 1972) and recently in man (Basch, 1972). The fact that testosterone is actively metabolized in human fetal liver to steroids with the 5β configuration (Solomon *et al.*, 1970) and that they are known to stimulate heme or hemoglobin synthesis (Granick and Kappas, 1967; Gordon *et al.*, 1970; Gorshein and Gardner, 1970; Necheles and Rai, 1971; Mizuguchi and Levere, 1971) prompted us to study their role in heme synthesis in the human fetal liver. As the human fetal liver undergoes dramatic developmental changes, the hormone–target cell relationship is a very dynamic one and can be analyzed in two different ways. First, one can analyze the ability of hormones such as testosterone and its metabolites to stimulate heme synthesis in physiological concentrations, and, second, one can investigate the sensitivity of the erythroid cells toward the inducers at different stages of development. In the first part of this investigation the characteristics of the tissue culture system were analyzed with respect to heme synthesis and its stimulation by steroids, both in primary cultures rich in erythroid cells and in fibroblast-like cells present in subcultures. In the second part of the study, the system of primary cultures, which is closest to the *in vivo* situation and very active in heme synthesis, was further analyzed along the lines outlined above, namely the comparison among testosterone and its metabolites, 5β- and 5α-androstanediol, with other erythropoietic active compounds and the study on the sensitivity of cells toward the hormones according to their state of development. Part of the results were presented at the meeting of the Canadian Society of Clinical Investigation (Congote and Solomon, 1972).

Experimental Procedure

Materials. $N^6,O^{2'}$-Dibutyryladenosine 3′,5′-monophosphate, cycloheximide, ethidium bromide, and actinomycin D were purchased from Sigma; 4-[^{14}C]testosterone (56.4 mCi/mmol), 7-[^{3}H]testosterone, 7-[^{3}H]androstenedione, and 1,2-[^{3}H]etiocholanolone were purchased from New England Nuclear Corp., and each had a radiochemical purity of at least 97% as judged by reverse isotope dilution analysis. Human erythropoietin, specific activity 36.3 units/

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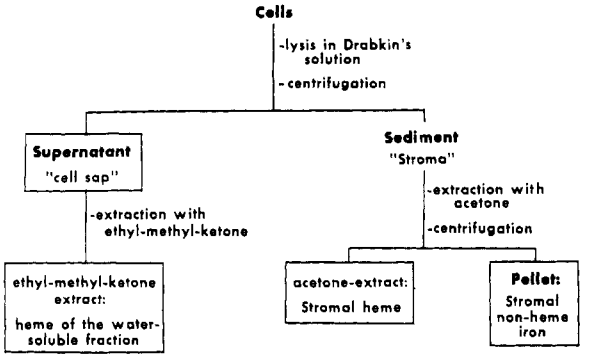

FIGURE 1: Outline of scheme employed in the lysis and fractionation of the liver cells.

mg of protein, was provided by the National Institutes of Health. It was collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina, and further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under Research Grant HE-10880. $^{59}FeCl_3$ (specific activities used: 7–22 mCi/mg of iron) was purchased from Frosst-Merck Laboratories, Montreal, and 2-[^{14}C]glycine (specific activity 57 mCi/mmol) from Amersham-Searle. All the materials used for tissue culture were purchased from Flow Laboratories, Rockville, Md., with the exception of Hank's basal salt solution, trypsin-EDTA, and chick serum which were purchased from Gibco. α-Amanitin was a gift from Professor Th. Wieland, Max-Planck Institute, Heidelberg, Germany.

Preparation of Cell Cultures. Cells were prepared from fetal livers and other tissues obtained at hysterotomy for therapeutic abortions at 8–20 weeks of gestation. All data concerning gestational ages were calculated from the crown-rump length using Patten's table (1968). The cell isolation was performed according to Kaighn's method (Kaighn and Prince, 1971) which is a modification of Coon's procedure for the isolation of rat hepatocytes (Coon, 1969; Cahn *et al.*, 1967). The tissues (liver or lung) were washed in Hank's balanced salt solution (Ca and Mg free) containing 200 units/ml of both penicillin and streptomycin. Then they were chopped in small pieces, washed again, and trypsinized in the same solution supplemented with 2% chick serum and 0.25% trypsin. After treatment for 15 min at 37° with stirring the cell suspensions were passed through cheesecloth and centrifuged at 400*g* for 15–20 min. The undigested pieces were trypsinized for a further 15 min with a fresh trypsin solution. The cells of both trypsinization steps were combined and suspended in a modified F-12 medium, containing double amounts of amino acids and sodium pyruvate, 1.5 g/l. of additional $NaHCO_3$, 50 units/ml each of penicillin and streptomycin, and supplemented with 17% fetal calf serum (Coon, 1969). Lung cells were prepared in the same way but only the trypsinization step of 30 min was used (Gielen and Nebert, 1971). Cells were plated to a density of about 2×10^5 cells/ml in Falcon tissue culture dishes of 6 cm diameter and were incubated at 37° and 5% CO_2 in humidified air. Subcultures were prepared by trypsinization with 0.05% trypsin and 0.02% EDTA in a modified Puck's saline A solution (Gibco). All experiments described using subcultures refer to cells which had undergone five–ten passages. The medium was changed twice a week. For the study of heme synthesis using primary cultures, the cells were incubated overnight after trypsinization and the next day the medium was changed to an L-15 medium (glycine-rich and iron-free) supplemented with 10% fetal calf serum (Bissel and Tilles, 1971) and the inducers to be tested. When steroids were used they were dissolved in ethanol–propylene glycol (7:3); dibutyryladenosine 3′,5′-monophosphate and erythropoietin were dissolved in the incubation medium and sterilized by filtration through nitrocellulose filters of 0.22-μ diameter. All incubation media, including controls, contained the same concentration of the ethanol–propylene glycol mixture, namely 0.05%. Subcultures were treated in the same way. For incubations longer than 24 hr the medium was changed every day.

^{59}Fe Incorporation and Heme Synthesis. For these studies we have essentially used the technique of Krantz *et al.* (1963). $^{59}FeCl_3$ was incubated overnight with human serum or heparinized plasma at 37° in order to bind it to transferrin. Then it was added to the cultures (5 μCi in 200 μl of plasma per dish) maintained in 3 ml of L-15 medium containing 10% fetal calf serum and incubated for various periods of time (normally 24 hr). An incubation time of 24 hr was found to be optimal for ^{59}Fe incorporation into subcultures. With primary cultures a very short incubation time was needed to get sufficient incorporation of radioactivity for a reliable measurement of heme synthesis. As we were interested in the comparison between primary cultures and subcultures, the same 24-hr time period was used in both systems. For studies of 2-[^{14}C]glycine incorporation into heme the same incubation conditions were used but the very glycine-rich L-15 medium was replaced by the glycine-free minimum essential medium dissolved in Hank's salt solution supplemented as before with 10% fetal calf serum. The glycine-free medium was used only in the 2-[^{14}C]glycine incorporation studies. The incubation medium contained 3.3 μCi of glycine/ml. After incubation the cells were washed three times with Hank's basal salt solution and lysed overnight in Drabkin's solution (1.5 ml per Petri dish) (Krantz *et al.*, 1963). After lysis the cell suspensions were separated into water-soluble and water-insoluble fractions by centrifugation at 700*g* (Figure 1). The water-soluble fraction was acidified with 0.13 vol of 1 N HCl and heme was extracted with ethyl methyl ketone (Krantz *et al.*, 1963). Iron incorporated into heme was separated from nonheme iron of the insoluble fraction by extracting it with acidified acetone (Rossi and Antonini, 1958).

In order to further analyze the nature of the radioactive iron or glycine incorporated in the ethyl methyl ketone extract, 50 mg of hemoglobin was added as carrier to the lysates of each dish; heme was extracted as before and crystallized twice as hemin according to the method of Labbé and Nishida (1957). Radioactivity was measured in a Packard liquid scintillation counter. The water-soluble residue left after the acetone extraction was dissolved in 1 ml of Nuclear-Chicago solubilizer and 11 ml of Instagel (Packard) prior to counting. Radioactivity of the water-soluble fraction was measured using the same scintillation mixture and, for the ketone extracts, Omnifluor (Packard) was employed. With the exception of the acrylamide electrophoresis data shown in Figure 2, all radioactive measurements were corrected for quenching and expressed as disintegrations per minute. For analysis of the heme proteins present in the water-soluble fraction by electrophoresis the fraction was first dialyzed for 48 hr with two changes of Drabkin's solution and for a further 24 hr against 0.04 M glycine, 0.05 M Tris-HCl, and 0.2 M sucrose (pH 8.3). The dialyzed fractions were layered on acrylamide gels prepared and run ac-

cording to the procedure described by Davis (1964) and human hemoglobin was added as a marker. After electrophoresis the gels were cut and slices extracted in 0.6 ml of water overnight and counted in 10 ml of Instagel (Packard). For the analysis of the heme-containing fractions the slices were shaken for 48 hr in 1.5 ml of Drabkin's solution and heme was extracted and counted as previously described.

Iron determinations in serum were done by the method of Trinder (1956) and proteins by the method of Lowry *et al.* (1951).

Metabolism of Testosterone. All details concerning the isolation of steroid metabolites, including extraction, chromatography, measurement of radioactivity, crystallization, and derivative formation, have been described elsewhere (Ruse and Solomon, 1966). The following paper chromatographic systems were used for the separation of the metabolites of testosterone: (A) toluene–propylene glycol; (B) cyclohexane–benzene (1:1)–propylene glycol; (C) Skellysolve C–propylene glycol.

Following crystallization to a constant $^3H/^{14}C$ ratio with added carrier, the final crystals and mother liquors were combined, a derivative was formed, and the product was further crystallized. The following derivatives were formed: testosterone was oxidized to androstenedione with CrO_3 in acetic acid; androstenedione was converted to testosterone by reduction with $NaBH_4$ and oxidation with the product formed with dichlorodicyanobenzoquinone; and etiocholanolone was reduced to 5β-androstanediol with $NaBH_4$.

A total of 290,000 dpm of 4-[^{14}C]testosterone was incubated either with primary or subcultured cells for 6–96 hr without changing the medium. The medium was then removed with a Pasteur pipet and partitioned between ether and water, and the organic phase evaporated to dryness *in vacuo*. In preliminary experiments, labeled metabolites present in the residue from the organic phase were identified by their mobility on paper chromatograms and in some instances by subsequent crystallization with added carrier. In later experiments described here metabolites were determined quantitatively by the addition to the ether extract of ^{3}H-labeled recovery markers and purification of each metabolite to a constant $^3H/^{14}C$ ratio. This experimental approach is the same as that employed by Mulay *et al.* (1972) to study the metabolism of testosterone in human skin fibroblasts.

The following is a description of a quantitative isolation of metabolites formed after a 6-hr incubation of [^{14}C]testosterone with a primary culture of human fetal liver cells. The aqueous phases following ether–water partition of the cells and the incubation medium contained 7700 and 5300 dpm, respectively, and were not further processed. To the ether extract of the medium (255,000 dpm of ^{14}C) was added 480,000 dpm of [^{3}H]testosterone, 56,000 dpm of [^{3}H]etiocholanolone, and 597,000 dpm of [^{3}H]androstenedione. The residue from this mixture was chromatographed in system A to the solvent front. Apart from a very small peak of radioactivity at the origin all the labeled material appeared in three separate peaks designated A, B, and C in order of decreasing polarity. Elution of the material in peak A which had the mobility of testosterone yielded a residue containing 246,700 dpm of ^{3}H and 61,300 dpm of ^{14}C. This material was chromatographed for 22 hr in system B where a single peak of radioactivity with the mobility of testosterone was observed 18 cm from the origin. The labeled material eluted from this zone contained 204,000 dpm of ^{3}H and 47,200 dpm of ^{14}C. The labeled material from peak

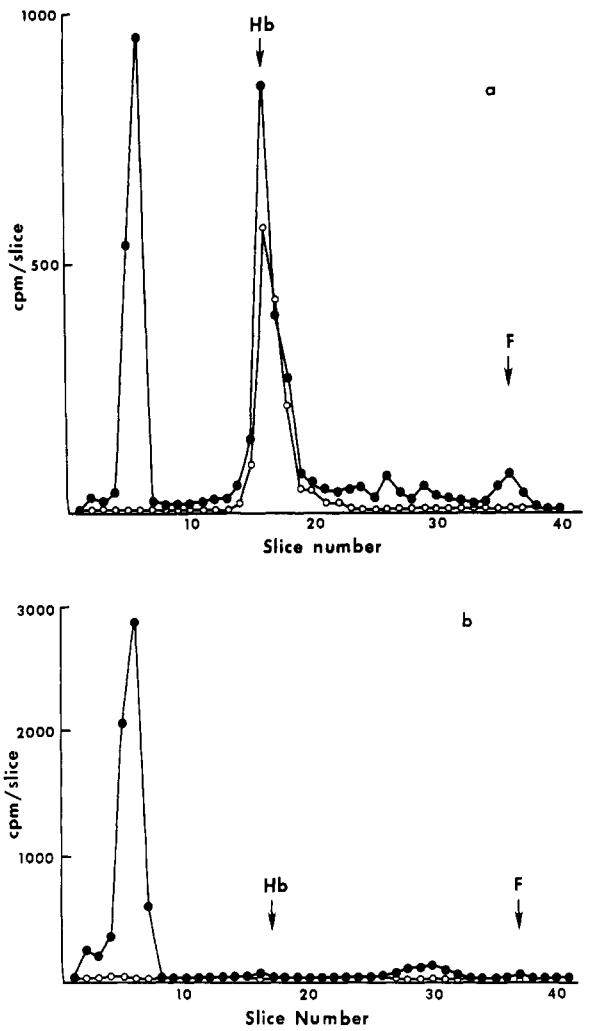

FIGURE 2: Polyacrylamide gel electrophoresis of the iron-containing proteins of the water-soluble fraction. Primary cells (1 day in culture) and subcultures of human fetal cells were incubated with ^{59}Fe and fractionated as indicated under Experimental Procedure. Primary cultures were incubated for 6 hr and subcultures were incubated for 48 hr in the L-15 medium prior to the addition of ^{59}Fe. With the exception of the instances indicated these conditions were used throughout the study. The water-soluble fraction was dialyzed first against Drabkin's solution and then against the electrophoresis buffer containing 0.2 M sucrose. The samples were run on acrylamide gels according to the method of Davis (1964) and the slices were analyzed for total ^{59}Fe content (closed circles) and heme (open circles). The arrows indicate the positions of hemoglobin marker (Hb) and the front (F), Bromophenol Blue dye: (a) data from primary cultures; (b) data from subcultures.

B, which had the mobility of etiocholanolone (11 cm from the origin), contained 63,050 dpm of ^{3}H and 12,300 dpm of ^{14}C. The excessive amount of tritium present in the etiocholanolone zone was due to incomplete separation from the area containing labeled testosterone. It was further purified by chromatography for 22 hr in system B whereupon a symmetrical peak of radioactivity was observed 35 cm from the origin. This material which had the mobility of etiocholanolone was eluted to give a residue containing 31,250 dpm of ^{3}H and 1700 dpm of ^{14}C. The material eluted from peak C (446,000 dpm of ^{3}H and 100,000 dpm of ^{14}C) was rerun in system C for 24 hr. A single peak of radioactive material with the mobility of androstenedione was observed 24 cm from the origin. The residue obtained after elution of this zone contained 384,000 dpm of ^{3}H and 80,000 dpm of ^{14}C.

The radiochemical purity of the metabolites isolated by two paper chromatographic systems was established in a

TABLE I: Iron Incorporation into Heme of the Water-Soluble Fraction (Ethyl Methyl Ketone Extract).[a]

Cell Type	Morphology	nmol of Fe/mg of Water-Soluble Protein
Primary liver cells 24 hr in culture	Mainly erythroid and epithelial cells (parenchymal)	11.0
Primary liver cells 48 hr in culture	Few erythroid, mainly epithelial cells	0.1
Subcultured liver cells	Fibroblastic cells	3.4
Primary lung cells 24 hr in culture	Several, mainly fibroblastic cells	0

[a] The cells present in one petri dish were incubated with 5 μCi of $^{59}FeCl_3$ for 24 hr at 37° and 5% CO_2 in medium L-15 supplemented with 10% fetal calf serum, washed, and fractionated as described under Experimental Procedure.

number of instances by crystallization to constant specific activity both before and after the formation of a derivative. The $^3H/^{14}C$ ratio of the final crystals did not differ significantly from that observed after the second chromatography. The chromatographic procedures employed were adequate to give a constant $^3H/^{14}C$ ratio and were indicative of the radiochemical purity for each metabolite. The above procedure was repeated for the incubation done at each time period. The percentage of each metabolite present in the ether extract was calculated from the final $^3H/^{14}C$ ratio, the amount of ^3H-labeled recovery marker added to the extract, and the total amount of ^{14}C present in the extract. With subcultured cells radiochemically pure androstenedione was the only metabolite isolated. The material having the mobility of etiocholanolone did not retain its radioactivity after crystallization with carrier steroid. In subcultured cells most of the radioactivity was accounted for by testosterone and androstenedione and a number of minor products whose identity could not be established.

Results

^{59}Fe Incorporation and Heme Synthesis in the Different Cell Types. The total incorporation of ^{59}Fe into the cells present in one petri dish was about 110,000–250,000 dpm. After incubation with ^{59}Fe and separation of the different cell fractions as indicated above, distribution of radioactivity was as follows: 19–30% in the water-soluble fraction; 23–36% as nonheme iron of the cell stroma; 41–49% in the acetone extract (heme of the water-insoluble fraction); and 0.8–3% in the ethyl methyl ketone extract (heme of the water-soluble fraction). These values represent the normal type of distribution of ^{59}Fe. The radioactivity of the ethyl methyl ketone extract can reach values as high as 24% when cells are plated in media supplemented with certain batches of fetal calf serum. As this fraction contains mainly hemoglobin the amount of radioactivity present is indicative of the number of erythroid cells remaining in the petri dishes. Because this latter fraction was expected to contain heme present in hemoglobin, we analyzed the nature of the ^{59}Fe incorporated in the methyl ethyl ketone extract in some detail. Using the same primary culture cells, three petri dishes were incubated with ^{59}Fe and three with 2-[^{14}C]glycine as detailed above. Then the heme of the ethyl methyl ketone extract was crystallized twice as hemin and the specific activity measured before and after crystallization. The specific activities, expressed as dpm of ^{59}Fe/mg of hemin, were 30,000 for the original extract and 27,000 after two crystallizations. The equivalent values for ^{14}C were 36,300 and 37,200 dpm/mg. These results show that the ^{59}Fe incorporated into heme of the methyl ethyl ketone extract corresponds to newly synthesized heme and not to other iron containing substances. ^{59}Fe incorporation was chosen over glycine incorporation because it is less expensive and allows an overall view of the iron distribution in the different cell fractions, which is of importance for the changes in iron metabolism in the cell during induction of heme synthesis. Table I shows the incorporation of iron into heme in the water-soluble fraction of the different cell types used in this investigation. Freshly isolated cells from fetal liver were very active in heme synthesis whereas cells in culture for 48 hr lost this ability to a very great extent. Subcultured cells show a greater activity with respect to heme synthesis as compared with long term incubations of primary cultures. In contrast to liver cells, fetal lung cells are not capable of synthesizing heme in the water-soluble fraction under the experimental conditions employed. Subcultured cells maintained in the L-15 medium used for the stimulation of heme synthesis are essentially nonproliferating in that there is a slight increase or no change in cell number during the 48-hr incubation period. They grow very rapidly if the L-15 medium is changed to the modified F-12 medium supplemented with 17% fetal calf serum described previously. The nature of the cells in the primary cultures is more difficult to analyze as they are a mixture which includes parenchymal cells and erythroid cells at different steps of maturation. The function of these different cell types under varying conditions of culture will be described (L. F. Congote and S. Solomon, manuscript in preparation). Studies are in progress to elucidate whether the cells that are active in heme synthesis are in a specific phase of erythroid cell differentiation and whether these cells are still dividing.

The nature of the heme formed in the water-soluble fraction was analyzed by electrophoresis on acrylamide gels (Figure 2). There were two main iron-containing proteins in the water-soluble fraction formed from primary cells (Figure 2a). The faster moving fraction had the same electrophoretic mobility as the hemoglobin marker and was the only protein containing newly synthesized heme. In contrast, less than 1% of the ^{59}Fe was associated with the nondialyzable material in the subculture and practically no hemoglobin was detected (Figure 2b). A total of 95% of the heme of the water-soluble fraction from the subcultures was in a freely dialyzable form. The nature of the slow moving protein seen in the electrophoretic pattern of both primary cultures and subcultures is now under investigation.

Effects of 5β-Androstanediol. Among the different steroids studied for their stimulation of porphyrin biosynthesis, those with the 5β-H configuration seem to be the most potent inducers (Granick and Kappas, 1967). As a result we began our studies using 5β-androstanediol, one of the main metabolites of testosterone in human fetal liver (Solomon *et al.*, 1970). In these initial studies we used a concentration of the steroid in the range of 10^{-5} M which was found to be optimal in the system described by Granick and Kappas (1967). Later we found that concentrations of the order of 10^{-7} to 10^{-8} M were also effective. Figure 3 shows the ^{59}Fe

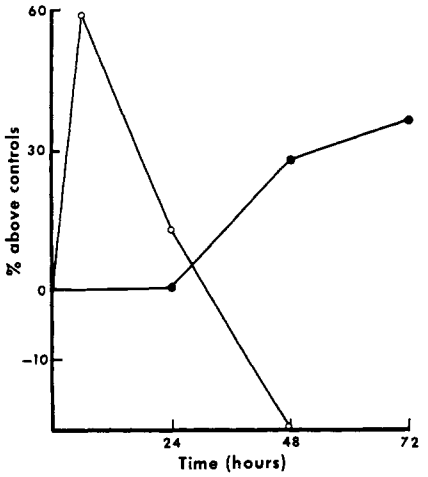

FIGURE 3: Time course of the stimulation of heme synthesis (ethyl methyl ketone extract) after treatment of the cells with 10^{-5} M 5β-androstanediol. After the indicated incubation times ^{59}Fe was added to the dishes and the iron incorporation into heme measured as indicated under Experimental Procedure. The results are expressed as per cent stimulation over the controls. The statistical analysis of the changes after short-term (6 hr) and long-term (48 hr) incubations with this steroid or testosterone will be shown in Tables III and IV: (○) primary cultures; (●) subcultures of human fetal liver cells. For the subcultures the control values at all time periods are given in Table I. For the primary cultures the control values were 25 nmol of Fe/mg of protein at time 0, 11 at 6 hr, 3 at 24 hr, and 0.4 at 48 hr.

incorporation into heme of the methyl ethyl ketone extract, expressed as per cent stimulation above control values after treatment with 10^{-5} M 5β-androstanediol. In this figure and in all subsequent tables we have indicated the incubation time with the particular steroid studied. After incubation, ^{59}Fe was added and the experiment stopped as indicated under Experimental Procedure. Optimal stimulation of heme synthesis by 5β-androstanediol in primary cultures was observed at the sixth hour of incubation. The steroid exhibits an optimal effect when the culture is relatively fresh and many erythroid cells are present. There is absolutely no stimulation in primary cultures after 48 hr of incubation and most of the cells present at this time are of epithelial morphology. 5β-Androstanediol can stimulate heme synthesis in subcultures but this effect can only be seen after a relatively long incubation period (Figure 3). It is of interest to note that after 48 hr of incubation there is no stimulation in primary cultures but a relatively high increase of heme synthesis in subcultures. We have investigated this further and have also analyzed other cell fractions obtained by the scheme outlined in Figure 1, and the results are shown in Table II. In subcultures, 5β-androstanediol stimulates not only heme synthesis in the water-soluble fraction but also in all other cell fractions studied. Primary cells incubated under the same conditions showed no significant increase above the controls and there are high variations from one experiment to the next. This is probably due to the different characteristics of cells from different fetuses and to the low ^{59}Fe incorporation into these cells as was seen in Table I.

Effects of Testosterone and 5β-Androstanediol on Primary Cultures after Short-Term Incubation. In order to determine whether stimulation of heme synthesis could be observed with physiologic concentrations of C_{19} steroids found in the circulation of human fetuses (Diez d'Aux and Murphy, 1972), short-term incubations were done with 5×10^{-8} M 5β-androstanediol. Because 5α-androstanediol was

TABLE II: Effect of 5β-Androstanediol on ^{59}Fe Incorporation into Different Cell Fractions.[a]

Cell Fraction	Primary Cells	Subcultures
Stromal heme (acetone extract)	7 ± 11 N.S.[b]	24 ± 5, $P < 0.01$
Heme of the water-soluble fraction (ethyl methyl ketone extract)	−20 ± 15 N.S.	51 ± 14, $P < 0.01$
Stromal–nonheme iron	10 ± 46 N.S.	35 ± 12, $P < 0.05$
Iron in cell sap	−16 ± 15 N.S.	41 ± 8, $P < 0.001$

[a] Primary cells and subcultures were incubated for 48 hr with 10^{-5} M 5β-androstanediol and then ^{59}Fe incorporation was done as described under Experimental Procedure. A total of 7 experiments were carried out with primary cultures and 11 with subcultures. The results are expressed as per cent of the controls ± standard error. The P values were calculated using the t test. The absolute values of the controls correspond to those indicated in Table I. [b] N.S. = not significant.

also found to be a metabolite of testosterone in the liver of the mid-term human fetus (Solomon *et al.*, 1970) this steroid was also tested. It was also of interest to test testosterone and dibutyryl-cAMP and to compare them to erythropoietin as there are a large number of erythroblasts in short-term incubations of fresh cells that could be stimulated by the latter hormone. These results are shown in Table III.

Of the steroids tested testosterone was the most active stimulator of heme synthesis in primary cultures and the 5α- and 5β-androstanediol had much lower activity. The stimulatory activity of testosterone was approximately the same as dibutyryl-cAMP and erythropoietin. In contrast to these data the stimulation of heme synthesis in subculture was greatest with 5β-androstanediol. Erythropoietin was not effective as the erythroblasts were not present in these cultures. 5β-Androstanediol is a better stimulator than testosterone, 5α-androstanediol, or dibutyryl-cAMP.

We next tested the effect of testosterone on iron incorporation into the different cell fractions and these data are shown in Table IV. The highest stimulation with testosterone was observed in the heme of the water-soluble fraction and small effects were seen in the other fractions tested.

Metabolism of Testosterone in Vitro. An example of the metabolism of testosterone in primary and subcultured cells as a function of time is shown in Figure 4. As can be seen the substrate disappeared within the first 24 hr of incubation in primary cultures. During this time the major product formed was androstenedione after which there was a gradual increase in the amounts of etiocholanolone formed. By contrast, subcultures of human fetal liver cells could not transform testosterone to etiocholanolone or any ring A reduced metabolites. Only small amounts of androstenedione could be detected following incubation with [^{14}C]testosterone.

Correlation of Testosterone Stimulation and Gestational Age. In the course of this investigation, which was done in large part with young fetuses, it was found that the response toward testosterone seemed to be dependent on the gestational age. For this reason we extended our study to older

TABLE III: Stimulation of Heme Synthesis in the Water-Soluble Fraction After a Short-Term Incubation.[a]

	^{59}Fe Incorpd into Soluble Heme		
	dpm/Dish	%	Subcultures, 48-hr Incubation, as % over Controls
Controls	3200 ± 140 (6)	0	0
Testosterone	8560 ± 1230 (6)	167	42 ± 23 (8)
5α-Androstanediol	4980 ± 960 (4)	55	34 ± 15 (8)
5β-Androstanediol	4530 ± 880 (4)	41	55 ± 22 (12)
Dibutyryladenosine 3′,5′-monophosphate	8010 ± 1610 (6)	150	46 ± 35 (8)
Erythropoietin	8800 ± 1230 (6)	175	No stimulation

[a] The same experimental procedure was employed as described in Figure 3 and Table II, but the time of incubation was 6 hr and the concentrations used were: testosterone and 5α- and 5β-androstanediol, 5×10^{-8} M; dibutyryladenosine 3′,5′-monophosphate, 5×10^{-7} M (Bottomley *et al.*, 1971); erythropoietin, 1.2 U/ml; fetal age 10–13 weeks. The per cent values over controls are shown. In the last column of this table the results obtained with subcultured liver cells are shown as a comparison with the effects seen with primary cultures. The subcultured cells (5–10 passages) do not come from the same fetuses used for primary cultures; means ± standard error; number of experiments in parentheses. The absolute values of the controls correspond to those in Table II.

TABLE IV: Effect of Testosterone on Iron Incorporation into Different Cell Fractions.[a]

Cell Fraction	% over control ± S.E.
Stromal heme (acetone extract)	39 ± 9 (18), $P < 0.001$
Heme of the water-soluble fraction (ethyl methyl ketone extract)	93 ± 20(18), $P < 0.001$
Stromal nonheme iron	30 ± 8 (16), $P < 0.002$
Iron in cell sap	55 ± 13 (18), $P < 0.001$

[a] The experimental procedure used was identical with that described in Table III except that the incubation time was 6 hr and 5×10^{-8} M testosterone was used. The results are expressed as per cent over controls ± standard error. The number of experiments performed is shown in parentheses. The P values were calculated using the t test. Primary cells were prepared from fetuses at 10–13 weeks of gestation.

fetuses, which are seldom available, and to the very young fetuses, where a small number of cells could be prepared from each fetus. When the effects of testosterone are expressed as a function of gestational age, the hormone is active only at 10–13 weeks (Figure 5). The highest effect seems to be in those cell cultures derived from fetuses with a crown-rump length of 59–71 mm (10–11 weeks). The effect is more pronounced in the heme from the water-soluble fraction, but the effects of testosterone on the iron incorporation in all other cell fractions show a similar correlation with the age of gestation. There were no significant differences in the absolute values of ^{59}Fe incorporation into heme in the different gestation groups (I–IV). The total number of cells used in the different age groups was the same but we did not have any data concerning the distribution of erythroid cells of different maturation states in these groups.

Effects of Inhibitors of RNA and Protein Synthesis. If the stimulation of heme synthesis by steroids is an induction process with *de novo* RNA and protein synthesis, this stimulation should be reversed by inhibitors of transcription or translation. Some of these inhibitors can be toxic for the cells and since long term incubations are necessary in subcultures, we have used them in very low concentrations. Inhibitors were added along with the steroids tested. Actinomycin D and cycloheximide were used in concentrations similar to those used by Nebert and Gielen (1971) in cultures of rat liver. α-Amanitin was used in a concentration found not to affect the total iron incorporation into the cell. Ethidium bromide was used as indicated by Zylber *et al.* (1969) and Zylber and Penman (1969). As can be seen in Table III all inhibitors used, independent of their mode of action, depressed the steroid-mediated stimulation to control levels.

Discussion

The human fetal liver is a complex tissue composed of cells of different morphology and function. The main cell types present at midterm are erythroid and parenchymal. They also represent the main cell components of primary cultures prepared from this tissue. The lifetime of erythroid and parenchymal cells in culture is quite different. One day after plating both cell types are present, but most of the cells are erythroid and this is quantitatively manifested by a very active heme synthesis. Two days after plating most of the cells remaining on the surface of the culture dish are of epithelial morphology, resembling liver parenchymal cells. Erythroid cells have almost completely disappeared and heme synthesis is consequently very low. The synthesis of heme has been studied using the well-established method of ^{59}Fe incorporation in an ethyl methyl ketone extract (Krantz *et al.*, 1963). This corresponds to a *de novo* synthesis of the molecule as was evident from the constant specific activity with respect to ^{14}C and ^{59}Fe after repeated crystallization of hemin synthesized from [^{14}C]glycine and $^{59}FeCl_3$. The electrophoretic analysis of the heme proteins present in the water-soluble cell fraction from freshly isolated primary cells showed that practically all newly synthesized heme present was part of hemoglobin. The electrophoretic system used here did not allow us to differentiate between the various types of hemoglobin synthesized in primary cultures. It is known that fetuses of similar gestational ages synthesize mainly fetal hemoglobin, although some adult hemoglobin synthesis is already taking place (Wood and Weatherall, 1973). The synthesis of heme in epithelial-like cells present after 2 days in culture is very low (Table I). If those cells are subcultured, the epithelial morphology is lost and only fibroblast-like cells can be observed in the culture dishes. Kaighn and Prince (1971) have demonstrated that some of these fibroblasts retain the property of liver parenchymal cells by synthesizing albumin. We have found that these subcultured liver cells synthesize a low but significant amount of heme in the water-soluble fraction (Table I). This heme is freely dialyzable and not associated

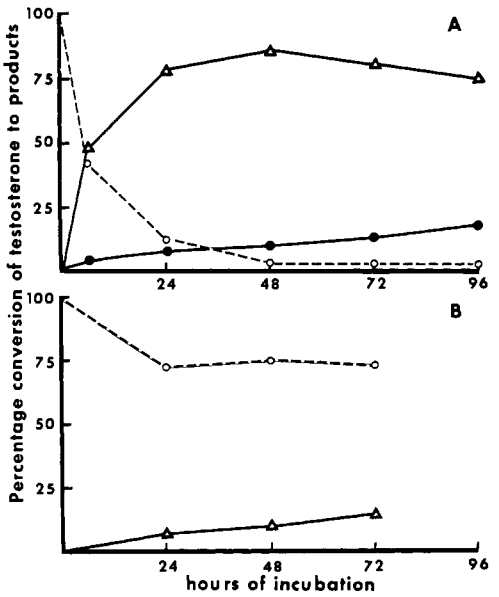

FIGURE 4: Formation of metabolites from [^{14}C]testosterone by cell cultures of huma fetal liver. Monolayer cultures of human fetal liver were incubated for varying periods of time with 290,000 dpm of [^{14}C]testosterone. To the ether extract of the medium were added ^{3}H-labeled testosterone, androstenedione, and etiocholanolone as recovery markers. Each metabolite was then purified to constant ^{3}H/^{14}C ratio: (O- - -O) testosterone; (●–●) etiocholanolone; (Δ–Δ) androstenedione. It was not possible to detect ^{14}C-labeled etiocholanolone in incubations with subcultures.

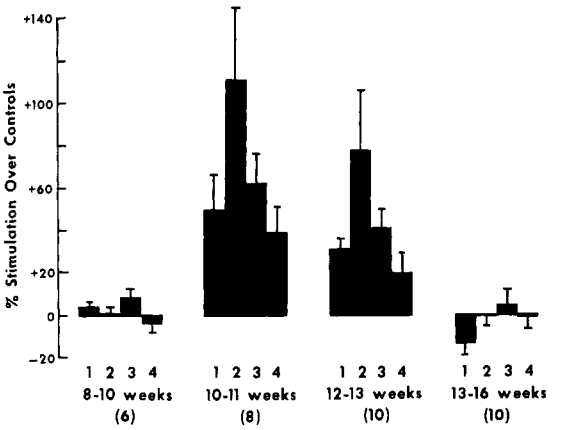

FIGURE 5: Correlation of gestational age and testosterone stimulation of iron incorporation into different fractions of primary cells. Experimental conditions are described in Table IV. The results are expressed as percentage stimulation by testosterone-treated cells over controls ± standard error. The number of determinations are shown in parentheses. The cell fractions were: (1) acetone extracts; (2) ethyl methyl ketone extract (soluble heme); (3) iron in the cell sap; (4) stromal non-heme iron.

with hemoglobin, as was the case in freshly isolated primary cultures. We have analyzed this system of heme synthesis in some detail in order to compare it with the same process in the erythroid cells of primary cultures.

Most of the known physiologic inducers of erythropoiesis that we have tested are active in both freshly isolated primary cultures (rich in erythroid cells) and in subcultured fibroblasts. The most important common characteristics in both systems will now be described. 5β-Androstanediol, one of the major metabolites of testosterone in human fetal liver at midterm (Solomon *et al.*, 1970), can stimulate heme synthesis in both systems. This stimulation is particularly pronounced in the heme from the water-soluble cell fraction, but the iron incorporation into all other cell fractions is also increased (Table II). A similar conclusion can be drawn concerning testosterone stimulation of iron incorporation in primary cells (Table IV). In primary cultures testosterone is by far the most active steroid tested (Table III). This means that in the system of primary cultures of human fetal liver the 5β-H configuration is not as critical as that described for other systems (Granick and Kappas, 1967; Gordon *et al.*, 1970; Gorshein and Gardner, 1970; Necheles and Rai, 1971). 5β-Androstanediol seems to be the most active steroid in subcultured liver fibroblasts but in comparison with 5α-androstanediol the difference is not very impressive. Testosterone, the most active steroid tested so far, is rapidly metabolized *in vitro* (Figure 4) in primary culture and the same general pattern of transformations was found as that observed *in vivo* in human fetal liver (Solomon *et al.*, 1970). This means that there is a specific 5β reduction in primary cultures whereas in adult human liver there is reduction both to the 5α and 5β configurations (Stylianou *et al.*, 1961). The effects seen in primary cultures of human fetal liver are then different from those seen in other target tissues for androgens, where metabolites with the 5α configuration are the most active compounds formed (Wilson and Gloyna, 1970). In subcultured cells, testosterone is not metabolized to 5β- and 5α-reduced products. In subculture liver cells 5α- and 5β-androstanediol are almost as active as testosterone or dibutyryl-cAMP in the stimulation of heme synthesis (Table III). The concentrations of testosterone used in these studies were similar to those found in the cord blood of male human midterm fetuses (Diez d'Aux and Murphy, 1972). From our studies it is not possible to say whether there is a sex difference in the stimulatory response to testosterone as cells obtained from male and female fetuses seem to respond in the same manner to the hormone. The stimulation of heme production at midterm by testosterone appears to be one of the steps in the complex series of events of biological differentiation. It remains to be seen if the synthesis of a specific type of hemoglobin is preferentially stimulated by the steroid, as has been reported for the stimulation in synthesis of C type hemoglobin by erythropoetin in young goats (Barker *et al.*, 1973).

Another common characteristic of both primary and subcultured cells is that the steroid mediated enhancement of heme synthesis can be blocked with very low concentrations of two inhibitors of transcription, namely actinomycin D and α-amanitin (Table V). We do not interpret the results with α-amanitin as a specific inhibition of the nucleoplasmic DNA-dependent RNA polymerase (Seifart and Sekeris, 1969; Kedinger *et al.*, 1970), since it is known that given *in vivo* this polypeptide has a more general effect on several types of RNA (Niessing *et al.*, 1970; Tata *et al.*, 1972). However, the fact that the two inhibitors with a different mode of action added in low concentrations during short-term incubations of primary cultures can lower to control levels the testosterone-mediated increase strongly suggests that this process is dependent on *de novo* RNA synthesis. Since the erythropoietic process under study is rather complex and involves different generations of cells, it is not possible to say at this time whether the effect of inhibitors of transcription is confined to the macromolecular synthesis of a single cell type, nor is it possible to conclude if cell proliferation involving DNA synthesis is similarly affected.

It is interesting to note that other specific inhibitors such as cycloheximide, which blocks protein synthesis, complete-

TABLE V: Effects of Inhibitors on the Steroid-Mediated Enhancement of Heme Synthesis.[a]

Inhibitors	Ratio Steroid Treated/Control Cells: 5β-Androstanediol Subcultures	Ratio Steroid Treated/Control Cells: Testosterone Primary Cultures
No inhibitors	1.47 ± 0.17	1.60 ± 0.20
Actinomycin D	0.98 ± 0.22	0.99 ± 0.20
α-Amanitin	1.04 ± 0.07	1.11 ± 0.12
Cycloheximide	0.95 ± 0.28	Not tested
Ethidium bromide	0.95 ± 0.07	Not tested

[a] The iron incorporation into heme of the water-soluble fraction was measured in two different cell systems: (a) subcultured cells from human fetal liver after 48-hr incubation with 5×10^{-8} M 5β-androstanediol and (b) primary cultures after 6-hr incubation with 5×10^{-8} M testosterone. Inhibitors were added along with the steroids. The concentrations used were as follows: actinomycin D, 2×10^{-8} M; α-amanitin, 5×10^{-7} M; cycloheximide, 2×10^{-7} M; and ethidium bromide, 0.2 μg/ml. The results from four experiments were expressed as ratios steroid-treated cells/control cells ± standard error.

ly inhibit the 5β-androstanediol-mediated stimulation of heme synthesis (Table V). The same effect could be seen after incubation with ethidium bromide, which especially inhibits mitochondrial transcription (Zylber *et al.*, 1969; Zylber and Penman, 1969). Since the δ-aminolevulinic acid synthetase is a mitochondrial enzyme (Kurashima *et al.*, 1970), this may be an interesting system for studying the relationship between mitochondrial synthetic processes and heme synthesis (Beattie, 1971).

The main differences in the regulation of heme synthesis between freshly prepared primary cultures and fibroblasts found in subcultures are the following: primary cultures respond rapidly to 5β-androstanediol with a preincubation period of 6 hr, whereas in subcultured cells the increase in heme synthesis is only observed after a 48-hr incubation with the steroid (Figure 3). Erythropoietin, the most potent and specific erythropoietic stimulating factor known, is also the most active in the stimulation of heme synthesis in primary cultures, but is completely inactive in subcultures (Table III).

The similarity in stimulation of heme synthesis in primary cells after treatment with testosterone and erythropoietin should be pointed out, especially as it pertains to the gestational age of the fetuses used in these studies. There is increasing evidence that androgens may act on the hematopoetic stem cells by triggering those cells to enter the G_1 phase and consequently the S phase of the cell cycle (Byron, 1970, 1971; Hait *et al.*, 1972). Since the maturation of erythropoietic stem cells is a very complex event, there are many steps where stimulation is possible and the abovementioned inducers may not be acting at the same level. On the basis of their experiments in adult mice, Hait *et al.* (1972) have suggested that androgens may act on erythropoietic stem cells in the G_0 (or early G_1) phase of the cycle, thereby triggering the cells to enter the S phase. Since erythropoietin seems to act on the G_1 phase (Kretchmar, 1966) this would mean that androgens act one step earlier than erythropoietin. More recent evidence by Gorshein *et al.* (1973) has supported this general concept. It is interesting to note that in our system testosterone can stimulate heme synthesis only in cells derived from fetuses of 10–13 weeks of gestation (Figure 5), reaching a maximum in cells derived from the liver of fetuses of 10–11 weeks. Basch (1972) found that the highest stimulation of hemoglobin synthesis in erythropoietic cells derived from human fetal liver after erythropoietin treatment is at 14–18 weeks of gestation. A study of the role of testosterone and erythropoietin on nucleic acid synthesis in the steps prior to heme and hemoglobin synthesis may provide evidence for a difference in their mechanism of action. Notwithstanding its mechanism of action, it is evident that testosterone and its metabolites may play an important role in erythropoiesis in human fetal liver and other cell processes involving heme synthesis.

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Interaction of Angiotensin Peptides and of Amino Acids with *p*-Nitrophenyl Acetate†

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ABSTRACT: The interaction of *p*-nitrophenyl acetate (NphOAc) with angiotensin II (AII), seven analogs and four lower homolog peptides, was studied as a function of pH. Second-order rate constants were obtained for NphOAc reaction with the amino, imidazole, and phenoxyl groups of the peptides. Comparison with Brønsted relations obtained for amino acid and other model compounds indicated that the histidine side chain in AII is free to interact with NphOAc while the tyrosyl side chain is partially restricted. Interpretation of the data obtained for the amino groups was precluded by the large relative errors associated with these data.

The conformation of angiotensin II (AII)[1] in solution was first studied by Smeby *et al.* (1962), who proposed a helical model, and by Paiva *et al.* (1963), who favored a random coil. More recently, other models have been proposed, mainly based on data from esr spectra of spin-labeled AII homologs (Weinkam and Jorgensen, 1971), circular dichroism (Fermandjian *et al.*, 1971), hydrogen–tritium exchange (Printz *et al.*, 1972), and nuclear magnetic resonance (nmr) of protons (Fermandjian *et al.*, 1972; Bleich *et al.*, 1973; Glickson *et al.*, 1973), of ^{13}C (Zimmer *et al.*, 1972), and fluorine (Vine *et al.*, 1973). Of these models, only the β- and γ-turn structures proposed by Printz *et al.* (1972) have been clearly described in detail. However, they have not been supported by the nmr evidence obtained by Marshall *et al.* (1973).

In view of the conflicting models being proposed for AII conformation, we believe that it will be useful to obtain more information about the state of that peptide's polar side chains in aqueous solution. We have previously obtained evidence, from electrometric titrations, of interactions between the amino and carboxyl groups of the N-terminal Asp residue with the imidazole of His[6] and the C-terminal carboxyl group (Juliano and Paiva, 1974).

In order to gain further information about the reactivity of the amino, imidazole, and phenoxyl groups of AII in solution we have attempted to investigate the interaction of these groups with *p*-nitrophenyl acetate (NphOAc). This paper presents the results of an analysis of the pH dependence of the reaction of NphOAc with AII and several analog and homolog peptides (Table I). Results obtained with several amino acids and other model compounds are also presented.

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[1] Abbreviations used are: AII, angiotensin II; NphOAc, *p*-nitrophenyl acetate. Peptides were named according to the IUPAC tentative rules for naming synthetic modifications of natural peptides (1967), *Biochemistry 6,* 362.