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REGULATION OF FETAL LIVER ERYTHROPOIESIS

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SUMMARY

The liver is the main erythropoietic tissue of the human fetus at midterm. The regulatory mechanisms involved in red cell formation should explain three main aspects of fetal erythropoiesis. First, the initiation of erythropoiesis during the formation of the embryonic liver from the hepatic endoderm. Second, the conservation of a high erythropoietic activity in the fetal liver for several weeks until achievement of a fully developed bone marrow. And third, the switch mechanism from fetal hemoglobin to adult hemoglobin.

There is experimental evidence for the induction of red cell formation in cells of the hepatic mesenchyme due to the interaction of these cells with the liver parenchymal cells. The nature of the factors involved in this process remain to be investigated but it has been found that at least one of them may be erythropoietin, a protein actively synthesized in cultures of fetal hepatocytes. Erythropoietin has been found in the fetal serum of mammals but seems to play a role late in the erythropoietic development of the liver. Liver erythroid cells isolated from young fetuses (8–12 weeks of gestation) show a poor response towards erythropoietin when compared with cells isolated from older fetuses. This observation suggests the involvement of other factors different from erythropoietin at early gestation. One of these factors is testosterone which stimulates heme and hemoglobin synthesis in liver cells isolated from fetuses of 10–13 weeks of gestation. Another set of factors are the hormones which interact with cellular β-andrenergic receptors. These factors act to regulate red cell formation in cells of fetuses obtained at 8 and 11 weeks of gestation.

The switch mechanism from fetal to adult hemoglobin remains one of the main unresolved problems of fetal erythropoiesis. Erythropoietin does not change the ratios of fetal to adult hemoglobin in cell cultures of human fetal liver. Testosterone and estradiol added to these cells *in vitro* can change the ratio of both hemoglobins but since the change is small these steroid hormones may play only a secondary role in the switch mechanism of globin chains. The fetal lamb seems to have a switch mechanism different from the human because experimental bleeding (to increase erythropoietin concentration) elevates the adult hemoglobin levels. It has been suggested that in the sheep the regulation of adult hemoglobin synthesis ($\alpha_2\beta_2$) may be indirectly controlled by an imbalance of γ to α chain mRNA's, which may change from 2 to 1 at early gestation to a ratio of 1:1 after 100 days of gestation. If a similar mechanism is operative in man remains to be investigated.

The liver is the main site of red cell formation in mammals during fetal life. More than three quarters of this tissue represent erythroid cells. Many important aspects of fetal liver erythropoiesis have been studied in some mammalian species such as sheep, mouse and rat. In the last few years we have been studying this process in man, using an *in vitro* cell culture system of fetal livers. The livers were obtained from fetuses after therapeutic histerotomy, an operation which was carried out relatively often until late 1975. We will focus our attention on the results obtained in human fetuses but we will complement these results with observations done on other mammals and on adult bone marrow cells.

1. *Initiation of fetal liver erythropoiesis: the role of the liver parenchymal cell*

The formation of the liver in the human embryo has been described by Severn[1, 2]. The liver parenchyma proliferates as endodermal cords of cells from a diverticulum of the endoderm, called hepatic diverticulum. These cords grow and anastomose with mesenchymal cells of the septum transversum. There are two different theories on the formation of hepatic hematopoietic cells. Rifkind *et al.*[3] consider that erythroid cells are the product of an inductive interaction between the proliferating endodermal cells of the liver cords and the mesenchyme of the septum transversum. The second theory has been proposed by Moore and Metcalf[4], who consider that the liver erythroid cells come from precursor cells of the yolk sac which migrate into the liver. The inductive interaction between liver parenchymal cells and mesenchyme cells has been studied by Emura and Tanaka[5]. These authors found that the liver cells could induce the development of endothelia and erythroid cells in the mouse metanephronic mesenchyme in culture. We tested the possible role of liver parenchymal cells on erythroid cells using an *in vitro* cell culture system of human fetal liver [6]. Human fetal livers were washed under sterile conditions, chopped in small pieces, trypsinized and plated in tissue culture dishes [6, 7]. Erythroid cell function was monitored by measuring the incorporation of ^{59}Fe

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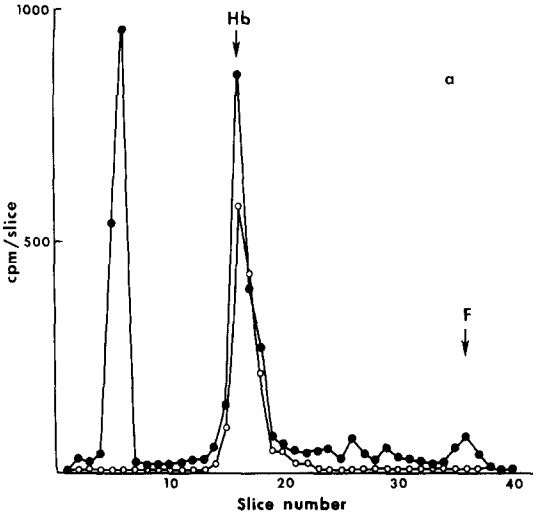

Fig. 1. Polyacrylamide gel electrophoresis of the iron-containing proteins of the water soluble cell fractions. Primary cells of human fetal liver were incubated with ^{59}Fe, lysed, centrifuged, and the proteins of the supernatant were analyzed on electrophoresis gels. The arrows indicate the position of hemoglobin (Hb) and the Front – bromophenol blue. Total ^{59}Fe is indicated with closed circles and ^{59}Fe associated with heme (extracted with ethyl–methyl–ketone) is indicated with open circles. This figure shows that the ^{59}Fe associated with heme in this fraction corresponds almost completely with hemoglobin. (from Congote *et al.*[7]).

into the heme fraction of water soluble hemoproteins. This fraction corresponds to heme being incorporated into hemoglobin (Fig. 1). When hemoglobin synthesis measured by this method is followed during different periods of cell culture of human fetal liver cells, one finds that on the first day of culture the synthesis is very high but disappears exponentially after two to three days. At this time the absence of erythroid cell activity corresponds to the rapid disappearance of the erythroid cells from the culture dishes, which are washed out in the culture medium leaving only monolayers of liver epithelial cells which have the morphology of parenchymal cells. If these cells are then incubated for two days in a fresh medium and this medium (conditioned by the liver cells) is used for the plating of fresh liver erythroid cells, one finds that the liver conditioned medium stimulates the ^{59}Fe incorporation into heme associated with hemoglobin (Table 1). This effect is remarkable because media conditioned with fetal kidney cells, the presumptive site of erythropoietin production, stimulated ^{59}Fe incorporation into heme to a lesser extent than the liver cells [6]. The liver parenchymal cells seem also to play an important role on the function of hepatic erythroid cells. It remains to be seen if their action consists in providing an optimal nutritional environment, in particular a good supply of iron [8]. Another possible role would be the production of factors which would increase the pool of erythroid cell precursors or accelerate red cell maturation. Using a very similar experimental approach for the culture of mouse fetal liver cells, Zucali *et al.*[9] have found that the factor produced by these cells is most probably erythropoietin because erythropoietin anti-sera suppressed the action of the liver conditioned media.

Table 1. Effect of kidney- and liver-conditioned media on heme synthesis in human liver erythroid cells

Media conditioned with	At a cell density of (cells/cm^2) $\times 10^4$	% Stimulation of heme synthesis ± S.E.M.
Kidney cells	2–4	24 ± 8 [6] $p < 0.05$
	4–6	14 ± 7 [6]
Liver cells	2–7	34 ± 13 [6] $p < 0.05$

Media were conditioned for 2 days with liver or kidney cells [6]. The effect of these media on hepatic erythroid cells was tested by plating about 10^7 cells of the latter in 1.5 ml of the conditoned media and measuring the ^{59}Fe incorporation into hemoglobin-associated heme. From Congote and Solomon[6].

2. *The appearance and disappearance of target cells for different hormones during fetal development*

It is possible to envisage a pool of target cells to erythropoietin remaining constant during fetal development. In this case the signal for the differentiation of these cells into mature erythrocytes would be the moment when erythropoietin begins to be synthesized in the fetus. The regulation of red cell formation is actually more complex because it is not only dependent on the presence or absence of a particular hormone but also on the presence of target cells. Basch[10] has found that the maximum effects of erythropoietin in hemoglobin synthesis in cell cultures of human fetal liver are seen in the cells prepared from fetuses between 16 and 18 weeks of gestation, whereas cells prepared from 6–10 week old fetuses showed a minimal response to the hormone. Since liver erythropoiesis begins at about the fifth week of fetal life and the synthesis of hemoglobin is very active at these early stages of fetal erythropoiesis, other hormones or erythropoietic factors different from erythropoietin are most probably involved in the regulation of liver red cell formation. We have been particularly interested in the role of androgens on this process. Granick and Kappas found ten years ago that metabolites of testosterone having the 5βH configuration were able to induce heme synthesis in chick embryo liver cells [11]. Further investigations by these authors and other groups demonstrated that androgens and their metabolites could stimulate heme and hemoglobin synthesis in a variety of tissues such as adult bone marrow cells, rat fetal liver cells and in cell cultures of chick blastodiscs [12–17]. Testosterone is synthesized in human male midterm fetuses [18]. This hormone is rapidly metabolized in the liver and the main metabolites are etiocholanolone and 5β-androstanediol, also steroids with the 5βH-configuration [19]. Testosterone is able to stimulate ^{59}Fe incorporation into heme associated

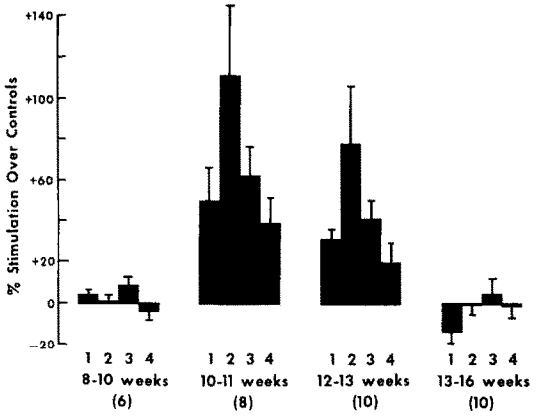

Fig. 2. Correlation of gestational age and testosterone stimulation of iron incorporation into different fractions of primary cells. The results are expressed as percentage stimulation by testosterone-treated cells over controls ± standard error. The number of determinations are shown in parentheses. The cell fractions were (1) acetone extracts (2) ethyl methyl ketone extract (soluble heme) (3) iron in cell sap (4) stromal non-heme iron (from Congote *et al.*[7]).

with hemoglobin in cell cultures of human fetal liver cells [7]. The maximal stimulation is seen in cells prepared from fetuses of 10–13 weeks of gestation, whereas cells isolated from fetuses of 8–10 weeks or 13–16 weeks of gestation do not show an increase of ^{59}Fe incorporation into heme after treatment with this hormone (Fig. 2). It seems also that a few weeks after the initiation of testosterone synthesis in the fetus there are target cells for this hormone in the population of liver erythroid cells, but these target cells apparently disappear after the 13th week of gestation. We have also found that isoproterenol, a potent stimulator of hematopoiesis in the mouse, was able to stimulate the synthesis of heme associated with hemoglobin in cells isolated from fetuses of 8–10 weeks of gestation but not afterwards (Congote and Solomon, *Endocrinology*, in press). The results described above with these three erythropoietic factors: erythropoietin, testosterone and isoproterenol (a drug

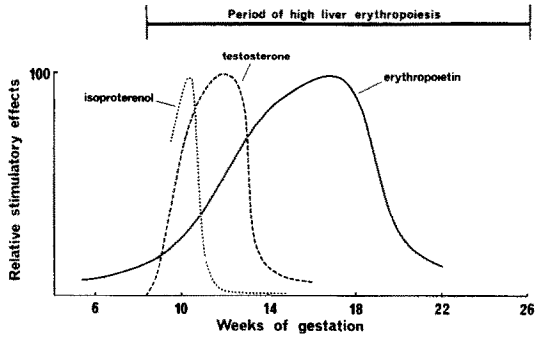

Fig. 3. Stimulation of hemoglobin synthesis or heme synthesis associated with hemoglobin in cell cultures of human fetal liver as a function of gestational age. The curve for erythropoietin was calculated from the published data of Basch[10], that for testosterone and isoproterenol from our own results ([7] and Congote and Solomon, *Endocrinology*, in press).

acting *via* cAMP) indicated that their target cells appear and disappear during different periods of hepatic erythropoiesis (Fig. 3). From these factors erythropoietin is certainly the most potent stimulator. For example, the effects of isoproterenol and erythropoietin are of the same magnitude in cells isolated from 6–10 week old fetuses and the effects of testosterone and erythropoietin are almost identical in cells prepared from 10–13 week old fetuses. The maximum effect of erythropoietin is seen only later, in fetuses between 16–18 weeks of gestation.

3. *The mechanism of action of erythropoietin*

The information available at the present time concerning the mechanism of action of erythropoietin comes from observations done both in adult bone marrow cells and on fetal liver cells. In mouse fetal liver cells the hormone increases both the number of precursor cells triggered to proliferate and differentiate to mature red cells and the number of cells originated from individual precursor cells [20]. Goldwasser and coworkers based their results on adult bone marrow cells. They suggest that erythropoietin interacts with specific receptors on the membrane of the erythropoietin-sensitive cell. This interaction results in the activation of a (so far poorly characterized) cytoplasmic factor which migrates to the nucleus and stimulates RNA synthesis [21]. The increase of RNA synthesis is the first detectable change in erythropoietin-sensitive cells after stimulation with the hormone [21]. This increase precedes and is independent of DNA synthesis. All types of RNA are stimulated [22]. Krantz and coworkers have found that erythropoiesis does not increase cAMP levels in fetal rat liver cells [23]. Since cAMP, dibutyryl-cAMP and drugs acting by a cAMP-mediated mechanism are potent stimulators of hematopoiesis [7, 24, 25], it is possible that this cyclic nucleotide may act synergistic to or may show a permissive effect to erythropoietin action in a variety of systems. Erythropoietin has been reported to stimulate cGMP levels in rat fetal liver cells [26] but not in adult bone marrow cells [21].

4. *The mechanism of action of androgens*

There is some confusion in the literature concerning the mechanism of action of androgens in erythroid cells. It is not clear if testosterone alone, or only its 5βH-metabolites, or both testosterone and its metabolites are active in these cells. Jepson, Gorshein, Gardner and coworkers propose that 5βH metabolites of testosterone induce the cycling of pluripotential stem cells, including those cells committed to erythroid differentiation [27]. Androgens would then act prior to erythropoietin by increasing the fraction of cells of the G_0 and G_1 phase which enter the DNA-synthetic phase of the cell cycle. Erythropoietin would then act on those cells committed to erythroid differentiation by triggering those cells to further division and maturation into erythrocytes. Both testosterone

and its 5βH metabolites have been reported to have this effect on the cycle of hematopoietic stem cells [16, 25]. Minguell and coworkers have been studying the mechanism of action of testosterone in rat bone marrow cells [28]. They suggest that the active steroid is testosterone itself, since there is no conversion of testosterone to 5αDHT or androstenedione in the rat bone marrow [29]. Furthermore, there is a nuclear receptor for testosterone in these cells [30]. Since it has not been possible to find a cytoplasmic receptor, the mechanism of action of testosterone on erythroid tissues may somehow be different from the usual model of steroid hormone action. Minguell *et al.* suggest that the target cells for testosterone are polychromatophilic erythroblasts [28]. This would mean that testosterone acts after erythropoietin in cells already involved in erythroid differentiation. The answer to these conflicting opinions about the target cells for androgens in erythroid tissues may be the presence of different target cells for these hormones in the erythroid cell line. Singer and Adamson[31] have found that etiocholanolone (a 5βH metabolite of testosterone) and fluoxymesterone (synthetic derivative of testosterone with androgenic properties) act on different populations of marrow cells which can be separated by velocity sedimentation on gradients of fetal calf serum. Independent of these uncertainties on the actual target of androgens, the molecular mechanism on the induction of hemoglobin synthesis seems to be essentially the same for testosterone and 5βH metabolites. The stimulatory effect of testosterone on heme synthesis in human fetal liver cells is blocked by simultaneous incubation with actinomycin D or α-amantin, indicating a possible role of RNA synthesis in this process (Table 2). Furthermore, testosterone stimulates the synthesis of a RNA fraction sedimenting at about 10–14*s* in sucrose density gradients in cell cultures of human fetal liver cells (Fig. 4). 5βH-metabolites of testosterone stimulate the synthesis of a RNA fraction of about 9*s* in cultures of chick blastodromes [17]. This RNA is probably involved in the synthesis of globin chains for fetal hemoglobin. The actual site of androgen action on the transcription process has not yet been elucidated.

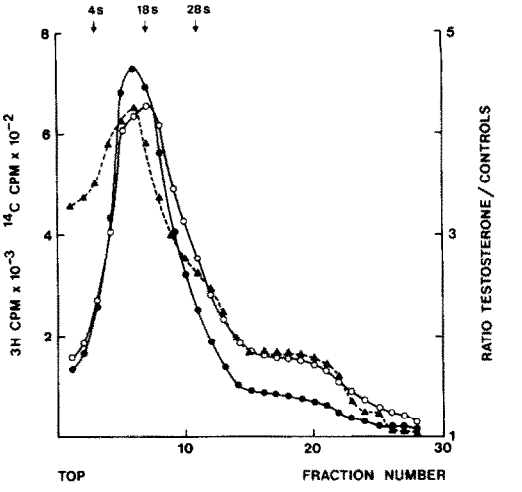

Fig. 4. Effect of testosterone on the sedimentation characteristics of rapidly labeled RNA isolated from erythroid cells of human liver. Two tissue culture dishes of 9-cm inner diameter containing liver erythroid cells were incubated for 5 h with L-15 medium alone (controls) or with L-15 medium containing 50 nM testosterone. A 1-h pulse with [^{14}C]-uridine (1.33 μCi/ml, controls) or [^{3}H]-uridine (13.3 μCi/ml, testosterone treated cells) was then done. At the end of the incubation, the cells of both dishes were pooled and the RNA was extracted and analyzed on sucrose gradient [32]. ○, controls; ●, testosterone-treated cells; △ ratio of testosterone/controls.

5. The switch mechanism from fetal to adult hemoglobin

About 90–95% of the total hemoglobin synthesized in the human fetal liver is fetal hemoglobin and the rest adult hemoglobin [33–35]. Fetal hemoglobin synthesis decreases dramatically after birth. The mechanisms involved in the switch from fetal to adult hemoglobin are still unknown. A knowledge of these mechanisms would probably allow the reactivation of fetal hemoglobin synthesis in adults suffering from sickle cell disease or β-thalassemia. This would alleviate or cure the anemic state of these patients. The elucidation of this problem is also of theoretical interest since the genes for β and γ chains are located in the same chromosome and very close to each other [36]. It is also possible that the two genes are transcribed as a single unit (polycistronic messenger). The β mRNA portion would be degradated or not translated in the fetus whereas the γ mRNA would be degradated or not translated in the adult. Lanyon *et al.*[37] have used the method of DNA-RNA hybridization to clarify this point. These authors have found that the amount of β and γ chains present in the fetal liver cells corresponds exactly with the amounts of mRNAs for β and γ chains and that there is not a polycistronic mRNA for β and γ mRNAs in the fetal liver or in the bone marrow. This means that even if the genes for β and γ chains are very close together in the same chromosome they are regulated independent from each other and this regulation takes place at the level of transcription. Kazazian *et al.*[38] do find, however, an excess of γ chain mRNA in midterm fetuses. These authors suggest that a decline in the γ to α chain ratio during fetal development would

Table 2. Effects of inhibitors on the testosterone-mediated enhancement of heme synthesis

Inhibitors	Ratio testosterone treated/control cells ± S.E.M. (n = 4)
No inhibitors	1.60 ± 0.20
Actinomycin D	0.99 ± 0.20
α-Amanitin	1.11 ± 0.12

From Congote *et al.* [7]. Experimental details explained in reference [7].

indirectly increase the proportion of adult hemoglobin being synthesized in the erythroid cells.

The hormonal regulation of the switch mechanism from fetal to adult hemoglobin remains to be elucidated. Basch[10] was unable to find any change in the ratio from fetal to adult hemoglobins after treatment of human fetal liver cells with erythropoietin. We have found a small but significant change in the ratio of the fetal to adult hemoglobin in human fetal liver cells treated with testosterone and estradiol. Testosterone increased this ratio and estradiol had the opposite effect. These changes were not at the level of globin chain synthesis but probably at a later stage of the assembly of the hemoglobin molecule ([39], and Congote, Bruno and Solomon, submitted for publication). The factor or factors responsible for this switch mechanisms also remain to be investigated. Other hormones should be tested for their effects on fetal and adult hemoglobin synthesis such as HCG and Thyroxin, because high levels of fetal hemoglobin have been observed in cases of thyrotoxicosis and molar pregnancies [40, 41].

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DISCUSSIONS

Friesen. What is known about comparative aspects of erythropoiesis—is the liver involved in erythropoiesis in a number of other species, and if so in which species?

Congote. The liver is important as erythropoietic tissue only in mammals. In birds the main erythropoietic tissue during fetal development is the yolk sac. There are some red cells in the chick liver. However, they are not in contact with the parenchymal cells but in sinusoid vessels. Then the liver is only active on erythropoiesis in mammals but not in birds or amphibians.

Friesen. I wonder if later in the discussion some of the sheep experts might tell us at what stage in the sheep fetus the liver is involved in erythropoieses and what is known about its hormonal control. A second question for you is: what sort of concentrations of erythropoetin are known to occur in the human fetus in terms of say adult levels of erythropoetin?

Congote. The concentrations of erythropoietin can change considerably. I will refer to bioassay data, since the radioimmunoassays for erythropoietin do not seem to work that well so far. In normal human adults, the serum erythropoietin levels are in the order of 0.1–0.2 U/ml but they can go as high as 0.7 U/ml in males and are usually lower in females (C. D. R. Dunn *et al., Exp. Hemat.* **3,** (1975) 65). High levels of erythropoietin have been found in human fetuses (Finne, *Acta Paed. Scand.* **53** (1964) 269). On the contrary, the concentrations of erythropoietin in the fetal sheep are undetectable and when present, usually lower than 0.2 U/ml (see Kazazian *et al., Nature* **260** (1976) 67). Note added in proof: Zanjani could not detect erythropoietin in normal sheep fetuses. This observation could be explained by the fact that sheep fetuses are not polycythemic. (E. D. Zanjani, discussion in R. Lindemann. *Erythropoiesis*, K. Nakao, J. W. Fisher and F. Takaku, Eds. University Park Press, Baltimore (1975) p. 339).

Tamaoki. I understand that stimulation of haematopoiesis by 5β-reduced steroids is also taking place on the molecular basis such as transcription and translation for new protein synthesis. My question is, whether or not you find any 5β-reduced steroids or other related steroid during your culture.

Congote. Testosterone is metabolized very quickly in previable human fetuses. The main metabolites are etiocholanolone, which is a 5β-reduced steroid, and 5β-androstanediol (M. D. Stern *et al., J. clin. Endocr. Metab.* **40** (1975) 997). As far as our cell cultures are concerned, testosterone is metabolized very quickly. The first metabolite isolated after 6–24 h of culture is androstenedione and after 48 h one can find etiocholanolone.

Tamaoki. Did you find any cytosol receptor to 5β-reduced steroids?

Congote. We are doing this type of investigation now but we do not have enough results so far. I am not an expert on receptors but I would like to comment on several publications on this subject. Valladares and Minguell (*Steroids* **25** (1975) 13) could not detect any cytosol receptor for testosterone in rat bone marrow cells. They found a very specific testosterone receptor in the nucleus of those cells. It is important to point out that neither 5β- nor 5α-reduced androgens can compete with testosterone on its receptor. There are two other publications on this subject: Mainwaring's group found cytosol receptors for 5β-reduced steroids in chick blastoderm, an important erythropoietic tissue in birds (D. M. Spooner and W. I. P. Mainwaring, *Acta endocr. Supp.* **177** (1973) 181). Other investigators (S. E. Lame, A. S. Gidari and R. D. Levere, *J. biol. Chem.* **250** (1976) 8209) describe the cytosol receptor for the 5β-reduced steroid etiocholanolone in fetal chick liver. I mentioned before that the chick liver is not an erythropoietic tissue. However, 5β-reduced steroids are potent inducers of heme synthesis in this tissue. These authors find a specific cytosol receptor for etiocholanolone but the dissociation constant is extremely high (in the order of 10^{-6} M) when compared with the normal dissociation constants of steroid receptors.

Tamaoki. The last question is why you did not employ 5β-reduced steroids such as 5β-dihydrotestosterone in your interesting culture system rather than testosterone itself.

Congote We tested this possibility. Etiocholanolone is one of the few steroids which stimulates heme synthesis in cultures of human fetal liver cells. However, its effect is lower than that of testosterone. If etiocholanolone were the active metabolite of testosterone, one could hardly explain the fact that testosterone is more active when added directly to cell cultures *in vitro*. The most likely explanation would be to assume a different mode of action for testosterone and 5β-reduced steroids, either in the same cell or in different cell populations. Singer and Adamson (*J. cell Physiol.* **88,** (1976) 135) have in fact found that the target cells for etiocholanolone and the androgen fluoxymesterone can be separated in gradients by velocity sedimentation. The test used for detection of erythropoietic activity was erythroid colony formation on plasma clots. These results suggest that different target cells for testosterone and 5β-reduced steroids could be the answer to the different activity of testosterone and etiocholanolone in different tissues, including human fetal liver.

Naftolin. You showed an effect with very large doses of estrogen, have you tried lower levels?

Congote. You are referring to the effect of estradiol on the ratios from fetal to adult hemoglobins. We have tried a concentration of 5×10^{-8} M but we could not see any effect at all.