*STIMULATION OF HEMOGLOBIN SYNTHESIS IN CHICK BLASTODERMS BY CERTAIN 5β ANDROSTANE AND 5β PREGNANE STEROIDS**

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Communicated June 30, 1967

Previous studies on the hematopoietic tissue of the chick blastoderm by Levere and Granick[1, 2] have led to the following tentative interpretations and hypotheses regarding the control of hemoglobin synthesis. The presence of heme appears to be necessary for the globin polypeptide chain to be completed, possibly for its appropriate folding around the heme. The synthesis of heme is limited by a repressor-operator mechanism that controls the synthesis of the limiting enzyme, δ-aminolevulinic acid synthetase. The colorless erythroid cells of the blastoderm appear to contain nonlimiting amounts of stable ribosomes and stable messenger RNA for the synthesis of globin, yet no globin is made until heme is synthesized. In these cells all of the enzymes of the heme biosynthetic chain are at nonlimiting activities except the first enzyme, δ-aminolevulinic acid synthetase. Once the repressor-operator mechanism is derepressed by some as yet unknown *derepressor*, the messenger RNA for δ-aminolevulinic acid synthetase is made and this enzyme is synthesized. Then follows the synthesis of δ-aminolevulinic acid which is eventually converted to heme. In the presence of heme, globin can be synthesized. In summary, according to this hypothesis, the formation of hemoglobin awaits the appearance of an unknown derepressor that will permit the synthesis of the messenger RNA of δ-aminolevulinic acid synthetase.

The studies reported here on the control of hemoglobin synthesis in erythroid cells were prompted by earlier studies by Granick[3] on the control of heme synthesis in chick embryo liver cells. Treatment of these cells in culture with certain drugs and chemicals was found to result in an increased *de novo* synthesis of δ-aminolevulinic acid synthetase and, as a consequence, a marked increase in porphyrin formation. Later, it was found by Granick and Kappas[4] that certain 5β-H steriods which are derived metabolically from several hormonal precursors and intermediates in man could also induce an increase in porphyrin formation in chick embryo liver cells. These results then raised the question of whether the same steroid metabolites, acting on the erythroid precursor cells of the chick blastoderm, could also induce the synthesis of δ-aminolevulinic acid synthetase and thus result in the more rapid formation of hemoglobin.

This paper reports that the same steroids which induce enhanced porphyrin synthesis in chick embryo liver cells also induce a more rapid synthesis of hemoglobin in the erythroblasts of the chick blastoderm. It is possible that these, or related, steroids are the physiologic derepressors which have been postulated to cause the increased synthesis of δ-aminolevulinic acid synthetase.

Materials and Methods.—The chick blastoderm, de-embryonated and cultured *in vitro*, was used to study the effect of certain 5β-H C_{19} and C_{21} steroid metabolites on hemoglobin synthesis. As previously described,[2] when blastoderms are obtained before the sixth somite stage of embryonic

development, the hematopoietic mesoderm of the blood islands is at a level of development comparable to the hemocytoblasts of mammalian bone marrow. When these blastoderms are deembryonated and grown on a simple glucose-agar medium, there is inhibition of cell migration and maturation in all cells except those of the hematopoietic mesoderm. These hematopoietic cells differentiate into the embryonic line of erythrocytes as well as into the endothelial lining cells of ill-defined tubules which enclose these erythrocytes. Since the maturation from erythroid precursor to definitive erythrocyte occurs during a period of 20–36 hr of growth on agar, this tissue simulates a phased culture of erythroid cells. Thus one can use this tissue to study certain biosynthetic events as they are related to progressive maturation of the erythroblast.

All eggs used were of the White Leghorn breed obtained from Shamrock Farms, New Jersey. After incubation at 37° for 22 hr the yolk with its attached blastoderm was removed from the shell and placed in chick Ringer's solution. The blastoderm was removed from the yolk and vitelline membrane, and the embryo was dissected from the blastoderm. The blastoderm was then bisected into symmetrical halves. (Details of this procedure have been described previously.[2]) One half of the blastoderm served as control; the other half was treated with steroid or other test substances. Since the blood islands are arranged lateral and posterior to the embryo in a symmetrical "horseshoe"-like pattern, the two halves are comparable in all ways. Each half was placed on a medium of 1% agar in Earle's solution, contained in a small Petri dish. The steroid was applied by employing a nebulizer to spray a 2% solution of the steroid in propylene glycol over the surface of the blastoderm half. It was estimated, by weighing the sprayed solution and knowing the area occupied by the blastoderm, that the surface of each test half was exposed to approximately 10^{-10} moles of steroid. After 3 hr of incubation at 37° in an atmosphere of 5 vol CO_2 to 95 vol air this procedure was repeated. The control halves were sprayed only with propylene glycol. Following the second application, the blastoderms were incubated for an additional 18–19 hr. At the end of this time period each of the paired halves was removed from the agar. The amount of hemoglobin present in each half was determined by a spectrophotometric method which has been outlined in detail elsewhere.[2] In brief, each blastoderm half was homogenized in a solution of 1% digitonin in phosphate buffer, pH 7.4, in order to hemolyze the red cells. After centrifugation the supernatant solution containing the hemoglobin was diluted to a known volume and then divided into two equal portions. Each aliquot was placed in one of a pair of semimicrocuvettes and the amount of hemoglobin present determined by measuring the difference spectrum between ferrous hemoglobin (433 nm) in one cuvette against CO-ferrous hemoglobin (418 nm) in the other cuvette. A Cary model 14 recording spectrophotometer with a 0.1 optical density full scale slide-wire was used for the determinations.

The test and control blastoderm halves were comparable and there was no statistical difference in their total nitrogen content as determined by a micro-Kjeldahl technique. For this reason the hemoglobin present is reported per half blastoderm.

The activity of δ-aminolevulinic acid synthetase in the blastoderms was determined by the Marver *et al.*[5] modification of the technique of Urata and Granick.[6] The total DNA content of the blastoderms was measured by the diphenylamine reaction as outlined by Burton.[7]

Results.—Hemoglobin-inducing steroids: When blastoderms were treated with the 5β-H steroids listed in Table 1, the treated halves usually contained two to three times more hemoglobin than the untreated control halves. The effect of each of the steroids considered individually was statistically significant at the 95 per cent

TABLE 1

STEROIDS WHICH INCREASE HEMOGLOBIN FORMATION IN THE BLASTODERM

Steroid	Hgb content greater in steroid-treated blastoderm half	Average ratio hemoglobin (steroid/control)
Etiocholanolone (5β androstane-3α-ol, 17-one)	13 of 15	1.8
Etiocholandiol (5β androstane-3α, 17β-diol)	13 of 15	2.2
11-Ketopregnanolone (5β pregnane-3α-ol, 11,20-dione)	12 of 15	2.1
17α-Hydroxypregnanolone (5β pregnane-3α, 17α-diol, 20-one)	12 of 15	3.1
Pregnanolone (5β pregnane-3α-ol, 20-one)	12 of 15	2.4
Pregnandiol (5β pregnane-3α, 20α-diol)	11 of 15	2.3

See *Materials and Methods* section for details of experiment.

confidence level or better. For example, with etiocholanolone as inducing steroid, 13 of 15 test halves had more hemoglobin than the control halves (Table 1). Most significant was the fact that the steroids which induced the increased hemoglobin formation were the same steroids which were most active in inducing porphyrin synthesis in fetal chick liver cells *in vitro*.[4]

Noninducing steroids: In Table 2 are listed the steroids which were ineffective

TABLE 2

STEROIDS WHICH HAVE WEAK OR NO HEMOGLOBIN-INDUCING ACTION IN CHICK BLASTODERMS

Steroid	Hgb content greater in steroid-treated blastoderm half
Testosterone (Δ^4-androstene-17β-ol, 3-one)	10 of 15
Progesterone (Δ^4-pregnene-3, 20-dione)	10 of 15
Estradiol ($\Delta^{1,3,5,10}$-estratriene-3, 17β-diol)	7 of 15
Tetrahydrocortisone (5β pregnane-3α, 17α, 21-triol, 11,20-dione)	6 of 15
Cortisol (Δ^4-pregnene-17α, 21-diol, 3,11,20-trione)	8 of 15
Androsterone (5α androstane-3α-ol, 17-one)	7 of 15
(5α)Pregnanolone (5α pregnane-3α-ol, 20-one)	6 of 15
(5α)Pregnandiol (5α pregnane-3α, 20α-diol)	7 of 15
Pregnandiol glucuronide (5β pregnane-20α-ol, 3α-yl-β-D-glucosiduronic acid)	7 of 15

in inducing increased hemoglobin synthesis in the chick blastoderms. These include the 5α-H epimers of several of the inducing steroids listed in Table 1, C_{21}-hydroxylated steroids, the natural estrogen estradiol, and the glucuronide conjugate of 5β pregnandiol. Testosterone and progesterone had, at most, only weak capacity to induce hemoglobin formation. Here again, those steroids which were inactive or poorly active in hemoglobin formation in the chick blastoderm were also inactive or weakly active in inducing porphyrin synthesis in chick embryo liver cells.[4]

Inducing steroids increase δ-aminolevulinic acid synthetase in the chick blastoderm: Since δ-aminolevulinic acid synthetase is the limiting enzyme in heme synthesis, an increase in hemoglobin, induced by steroids, might be caused by an increase in the activity of this limiting enzyme. To test this possibility, the activity of δ-aminolevulinic acid synthetase was determined in pooled, steroid-treated halves and compared with pooled control halves. Because an increase in δ-aminolevulinic acid synthetase activity would be expected to precede maximal hemoglobin formation, the measurements were performed 18 hours after incubation on agar. In Figure 1 are shown the results with five control preparations and six experiments with three inducing steroids. It may be seen that control blastoderms had little or no measurable activity. In the blastoderms treated with etiocholandiol, 11-ketopregnanolone, and 17α-hydroxypregnanolone, the δ-aminolevulinic acid synthetase activity was clearly detectable.

A possible explanation for the increased δ-aminolevulinic acid synthetase activity in the steroid-treated blastoderms was that the steroids stimulated replication of the erythroid cells of the blastoderm and that there was a greater number of cells, all with normal enzyme activity. However, determination of the DNA content of steroid-treated as compared to control blastoderm halves revealed no significant differences. Therefore, the inducing steroids do not increase cell multiplication but rather cause an increase in the activity of δ-aminolevulinic acid synthetase per cell.

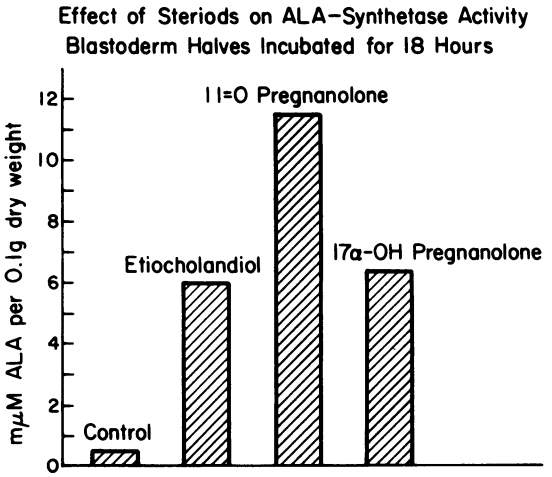

FIG. 1.—δ-Aminolevulinic acid (ALA) synthetase activity as determined on pooled blastoderm halves. Untreated blastoderms have little or no enzyme activity. Blastoderms treated with the steroids noted have definitely detectable δ-aminolevulinic acid synthetase activity.

The increase in activity of δ-aminolevulinic acid synthetase induced by the steroids could be caused by an increase in the *de novo* synthesis of the enzyme or by an increase in the activity of the enzyme already present. Actinomycin D in low concentrations prevents the formation of messenger RNA. Previous studies with actinomycin D[2, 8] have shown that hemoglobin synthesis in the blastoderm is limited primarily by the rate of synthesis of the messenger RNA that codes for the enzyme δ-aminolevulinic acid synthetase. When the blastoderms were treated with actinomycin D, a small amount of hemoglobin was formed, probably due to the presence in these erythroid cells of the preformed messenger RNA of δ-aminolevulinic acid synthetase and of the enzyme itself in these erythroid cells. In the present study when actinomycin D (0.1 μg/ml) was incorporated into the agar and the blastoderms were sprayed with 11-ketopregnanolone, the steroid-inducing effect was eliminated. At this concentration actinomycin D is not lethal for the cells. If the steroid had merely activated an inactive form of δ-aminolevulinic acid synthetase, then the treated blastoderms should have produced more hemoglobin than untreated controls even in the presence of antinomycin D. It is concluded from this experiment that the induction by active steroid requires the ability of the blastoderm erythroblasts to form messenger RNA and that the increase in activity of δ-aminolevulinic acid synthetase stimulated by steroids is a result of the *de novo* synthesis of this enzyme. The present result is consistent with observations on chick embryo liver cells[4] which showed that the stimulation of porphyrin synthesis by steroids was also blocked by actinomycin D.

In the presence of puromycin (5 μg/ml), which inhibits protein synthesis at the ribosome level, only trace amounts of hemoglobin were formed in the blastoderms. The addition of inducing steroid together with puromycin did not increase the yield of hemoglobin. These results are to be expected because puromycin not only

blocks the synthesis of δ-aminolevulinic acid synthetase but also blocks formation of globin.

Failure of chemicals which induce hepatic porphyria to enhance hemoglobin formation in erythroid cells: The chemicals allylisopropylacetamide and 3,5 dicarbethoxy-1,4-dihydrocollidine, both of which are potent inducers of hepatic porphyrin production in various animals and in chick embryo liver cells, were tested on chick blastoderms in concentrations known to strongly stimulate hepatic porphyrin synthesis.[3] These chemicals failed to stimulate hemoglobin formation in the erythroid cell cultures.

Discussion.—The stimulation of hemoglobin formation in erythroid cell cultures of the chick blastoderm represents a newly defined biological activity of 5β androstane and 5β pregnane steroids. Control of heme and hemoglobin synthesis in these cultures resides in the rate-limiting enzyme, δ-aminolevulinic acid synthetase,[1, 2] and steroids appear to act by inducing enhanced *de novo* formation of this enzyme. This conclusion is supported by finding an increase in this enzyme activity after steroid treatment unassociated with any change in total DNA content of of the blastoderms and also by the finding that actinomycin D eliminates the steroid effect on hemoglobin formation.

A degree of steric specificity is evident in the ability of steroids to induce the increased hemoglobin formation. A prime requisite is a 5β-H (A:B *cis*) or highly angulated A:B ring junction. Steroid metabolites of the 5α-H (A:B *trans*) or planar type did not induce hemoglobin formation in these cultures and primary hormones such as testosterone and progesterone had only weak activity. It is possible that any weak inducing activity that these primary hormones may possess reflects, in part, their conversion in culture to their more potent 5β-H metabolites.

It is of interest to note that foreign chemicals, such as allylisopropylacetamide and 3,5 dicarbethoxy-1,4-dihydrocollidine, which induce the enhanced formation of δ-aminolevulinic acid synthetase in liver cells have no effect on hemoglobin formation in erythroid cells while the 5β-H steroid hormone metabolities are active in both cell types. This suggests that regulation of heme synthesis in erythroid cells, as compared to that in liver cells, may reside in a more restricted control mechanism, possibly involving a more specific aporepressor which is responsive only to certain types of physiologic substances such as the inducing steroids described here.

The erythropoietic action of testosterone in man has been attributed to the increased renal erythropoietin output which is evoked by large amounts of this sex hormone. The present study demonstrates that 5β-H C_{19} and C_{21} steroids which lack classical sex hormone actions are capable of stimulating hemoglobin formation directly in erythroid cells by a mechanism not dependent on renal erythropoietin. The possibility may be considered, therefore, that part of the androgen action on erythropoiesis is mediated by direct bone marrow effects of active steroids of the types described in this study.

It is suggested as a working hypothesis (Fig. 2) that certain of the 5β-H C_{19} and C_{21} steroids, which have been found to stimulate hemoglobin formation in the erythroid cells of the chick blastoderm, may serve in the physiological regulation of hemoglobin production in man.

Summary.—Certain steroids stimulate two- to threefold the early formation of hemoglobin in erythroid cells of chick blastoderms cultured *in vitro*. These steroids are compounds of the C_{19} and C_{21} neutral type; share the basic nuclear structure

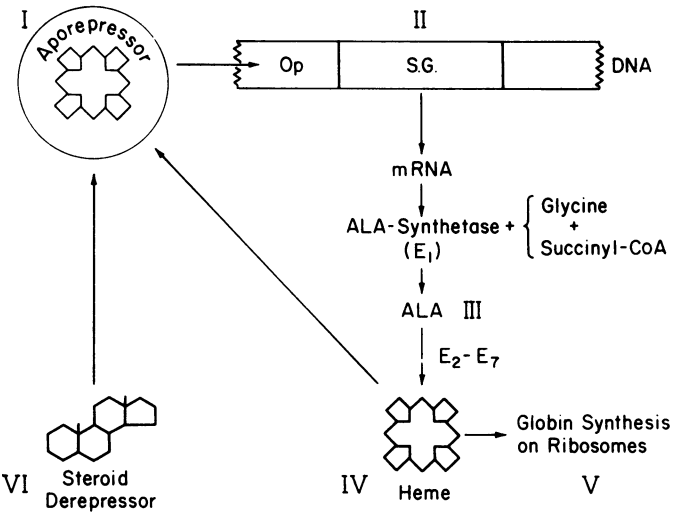

FIG. 2.—Hypothesis on the control of hemoglobin formation in blastoderm erythroblasts. (*I*) An aporepressor-corepressor (heme) operator (*Op*) mechanism controls the transcription of the structural gene (*S.G.*) *II* into messenger RNA of the limiting enzyme δ-aminolevulinic acid (ALA) synthetase (E_1). This enzyme combines the substrates succinyl-CoA and glycine to form ALA (*III*) which is further converted to heme (*IV*) by a series of nonlimiting enzymes (E_2–E_7). Heme is required for the completion of synthesis of the globin polypeptide chain (*V*). A steroid derepressor (*VI*) competes with heme for a site on the aporepressor. The combination of aporepressor and steroid deprives the repressor mechanism of effectiveness and permits transcription of the structural gene *II*, the formation of ALA, heme, and hemoglobin. None of the positions of the genes in the genome is known.

characteristic of 5β-H (A:B *cis*) compounds; have alcohol or ketone substituents at carbons 3, 11, 17, or 20; and are derived from the *in vivo* biotransformation of several precursor hormones, or intermediates, in man.

It is suggested as a working hypothesis that inducing steroids act as physiological derepressors to permit the structural gene for δ-aminolevulinic acid synthetase in erythroid precursors to be transcribed more readily. Since δ-aminolevulinic acid synthetase is the limiting enzyme in heme biosynthesis, an increase in this enzyme leads to the synthesis of more heme and, following this, of more hemoglobin. Support for this hypothesis is the finding of an increase in the activity of δ-aminolevulinic acid synthetase in the erythroid tissue after steroid treatment, and the finding that actinomycin D eliminates the steroid enhancement of hemoglobin formation.

* This work was supported, in part, by USPHS grants AM 09838 and G.M. 04922, and New York City Health Research Council grant U-1686. One of the authors (A. K.) was the recipient of a fellowship from the John Simon Guggenheim Memorial Foundation, 1966–1967.

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