Studies on the Control of Hemoglobin Synthesis: 
The in vitro Stimulating Effect of a 5β-H Steroid Metabolite on Heme Formation in Human Bone Marrow Cells

By THOMAS F. NECHELES AND UMA S. RAI

The effect of androgenic steroids on hemoglobin levels has usually been ascribed to an indirect effect of these hormones, possibly secondary to an effect upon erythropoietin production or activation.1,2 Earlier studies of the in vitro effect of androgens on bone marrow cells provided conflicting evidence for a direct effect upon erythroid cell proliferation or hemoglobin synthesis. Reisner3 presented evidence for an in vitro effect upon iron incorporation by canine bone marrow cells whereas other workers were unable to confirm these results.4 In earlier studies, we could not document an in vitro effect of testosterone on hemoglobin synthesis, nucleic acid synthesis, or erythroid cell proliferation. Recently Granick and Kappas and their co-workers5-7 have published a series of papers in which they report a potent stimulation of heme synthesis in embryonic chick liver and erythropoietic tissues using certain metabolic derivatives of androgenic steroids. We have now demonstrated a similar in vitro effect of 5β-H metabolites of testosterone in adult human erythropoietic tissue. These data add support to the concept that androgenic hormones or their metabolic products may act directly upon human red cell precursors.

Methods

Bone marrow was obtained from normal young adults of both sexes by aspiration, anticoagulated with heparin (15 IU per ml. marrow aspirate) and the nucleated cells concentrated by differential centrifugation at 4°C. The nucleated cells were resuspended in a medium consisting of one-third autologous plasma, two-thirds Hanks balanced salt solution.8 Penicillin G was added to a final concentration of 100 units/ml. The appropriate steroid was dissolved in absolute ethanol. Control flasks received an equivalent quantity (0.1 ml.) of absolute ethanol. Duplicate flasks, containing 15 × 10⁶ nucleated bone marrow cells in a final volume of 3.0 ml and 1 microcurie of glycine-2-H₃,* were incubated under 5 per
cent CO₂ in air at 37 C. for 24 hours. The cells were then washed three times with cold saline, exposed to hypotonic buffer for 30 seconds, and isotonicity reestablished by the addition of one-fifth volume of 1.5 M sucrose solution. This procedure disrupted erythroid precursors while leaving intact the nonerythroid elements. One hundred milligrams of carrier hemoglobin was added to each flask and intact cells, nuclei, and cell stroma were removed by centrifugation. Globin was precipitated with 9 volumes of ice-cold acid-acetone (1.4 per cent HCl in acetone) and heme crystallized from the supernatant. Both heme and globin were purified by repeated washing and reprecipitation. The globin was then redissolved in performic acid and an aliquot taken for protein determination. The heme was dissolved in acetone and the concentration determined. Aliquots were dried, combusted in oxygen, the titrated water absorbed in ethanol, and counted in a liquid scintillation counter with an efficiency of thirty per cent and a preset count of 10,000. Each sample contained at least one milligram of heme or ten milligrams of globin. The data were expressed as disintegrations per minute per milligram of heme or globin. Delta amino-levulenic acid synthetase activity was estimated by the technic of Vavra and co-workers. Hemolysates were prepared from washed bone marrow cell suspensions which had been incubated for 24 hours with and without the appropriate steroid. One-half the hemolysate from each sample was incubated for 90 minutes with 14C-labeled glycine, whereas the other half was incubated with Delta amino-levulenic acid-4-14Cl. Fortification of the hemolysate with either pyridoxal phosphate or succinyl-Co-enzyme A, or a combination of the two, failed to significantly stimulate heme synthesis under these conditions. After 90 minutes, heme was isolated and counted in a manner similar to that described above.

**RESULTS**

Two related steroid metabolites were used; etiocholanolone† (5 β androstane-3 α-ol, 17-one) and androstanolone‡ (5 α androstane-17 β-ol, 3-one). Androstanolone was ineffective in stimulating the incorporation of glycine-H₃ into either heme or globin in this system (Table 1). The addition of etiocholanolone, in constrast, led to a consistent and significant stimulation of glycine-H₃ incorporation into heme. Stimulation of glycine incorporation into globin was more variable. No stimulation of heme synthesis could be observed at two hours and an incubation period of 24 hours proved to be an optimal time to observe this effect. Following longer period of incubation, the results became more variable. The optimal range of concentrations proved to be rather narrow, about 3 x 10⁻⁸M (Fig. 1). Bone marrow suspensions from both male and female donors responded to a similar degree.

The effect of metabolic inhibitors on heme synthesis was studied in one series of experiments using actinomycin D* and puromycin* (Table 2). Addition of either compound led to a moderate decrease in glycine-H₃ incorporation into heme in the control flasks. Furthermore, both compounds abolish completely the stimulating effect of etiocholanolone. Delta amino-levulenic acid (ΔALA) synthetase activity was estimated by comparing the incorporation of glycine-2-C¹⁴ and ALA-C¹⁴ into heme (Table 3). ΔALA incorporation was unchanged in cells preincubated with etiocholanolone. Glycine-C¹⁴ incorporation, in contrast, was markedly stimulated in the presence of the 5 β-H steroid. Control experiments in which the steroid was added just prior to

---

*Specific activity-1mc/mM, obtained from New England Nuclear Corp. Boston, Mass.
†Specific activity-1mc/mM, obtained from New England Nuclear Corp. Boston, Mass.
‡Obtained from Sigma Chemical Corp., St. Louis, Mo.
Table 1.—The Effect of Certain Steroids upon the In Vitro Synthesis of Heme and Globin

(Results expressed as DPM/mg.)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>Androstaneolone 3α-ol, 17-one (3×10⁻⁸M)</th>
<th>Etiocholanolone 17β-ol, 3-one (3×10⁻⁸M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>heme</td>
<td>globin</td>
</tr>
<tr>
<td>1</td>
<td>1196</td>
<td>523</td>
<td>1117</td>
</tr>
<tr>
<td>2</td>
<td>1200</td>
<td>254</td>
<td>872</td>
</tr>
<tr>
<td>3</td>
<td>681</td>
<td>340</td>
<td>721</td>
</tr>
<tr>
<td>4</td>
<td>2765</td>
<td>905</td>
<td>5548</td>
</tr>
<tr>
<td>5</td>
<td>356</td>
<td>407</td>
<td>1069</td>
</tr>
<tr>
<td>6</td>
<td>2974</td>
<td>401</td>
<td>8678</td>
</tr>
</tbody>
</table>

p value N.S. N.S. <0.05 0.1

The statistical analysis was carried out on paired samples using the control value from each experiment.

Discussion

These results extend to human erythroid cells the findings of Levere and co-workers using erythroid tissue of the chick blastoderm. These authors showed that certain steroids of 5β-H type, with a highly angulated nucleus, stimulate heme formation in embryonic chick erythroid cells, whereas similar steroids...
CONTROL OF HEMOGLOBIN SYNTHESIS

Table 2.—Effect of Metabolic Inhibitors on Heme Synthesis

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Incubated with Etiocholanolone</th>
<th>± 1 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>Control</td>
<td>1598 ± 120</td>
</tr>
<tr>
<td>+ Actinomycin D (1.5 µg./ml)</td>
<td>1061 ± 110</td>
<td>3045 ± 352</td>
</tr>
<tr>
<td>+ Puromycin (15 µg/ml)</td>
<td>900 ± 81</td>
<td>911 ± 91</td>
</tr>
</tbody>
</table>

Table 3.—Δ ALA Synthetase Activity

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Preincubated with Etiocholanolone</th>
<th>DPM/mg heme (± ISD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine — C14</td>
<td>Control</td>
<td>10548 ± 807</td>
</tr>
<tr>
<td></td>
<td>12018 ± 520</td>
<td>17846 ± 269</td>
</tr>
<tr>
<td>Δ ALA — C14</td>
<td></td>
<td>14189 ± 1422</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15635 ± 1433</td>
</tr>
</tbody>
</table>

The mean ± SD of two separate experiments, each carried out in triplicate.

with a planar nucleus (5 α-H type) failed to exhibit this activity. The parent compound, testosterone, had no effect when added to either chick embryonic cells or adult human bone marrow cell suspensions. Stimulation of heme synthesis in erythroid tissue, therefore, may be induced by a group of compounds which previously had been considered to be metabolic by-products of the active parent compound. These metabolic by-products are normally present in only trace amounts and their biologic activity has in general been discontinued.

The action of the metabolic inhibitors, actinomycin D and puromycin, in the control flasks, suggests that heme synthesis in this system is dependent, to some extent, upon continued RNA and protein synthesis. The finding that steroid-induced stimulation of heme synthesis is completely abolished by both of these inhibitors suggests that the stimulatory effect is mediated by newly formed RNA-dependent protein synthesis rather than directly upon performed enzymes.

It has appeared from results of a number of studies\textsuperscript{10–12} that heme may play a central role in the control of hemoglobin synthesis in the erythroid precursor. Inhibition of heme biosynthesis, especially by feedback inhibition, limits the formation of globin. An excess of ΔALA, by circumventing the rate-limiting point in the heme biosynthetic sequence, stimulates globin formation. The biosynthesis of hemoglobin is thus regulated, at least to some extent, by the activity of the enzyme ΔALA synthetase.\textsuperscript{13} Levere and co-workers\textsuperscript{5} have previously reported an increase in this enzyme activity in chick embryonic erythroid cells treated with 5 β-H steroid. ΔALA synthetase activity can be estimated in hemolysates by comparing the incorporation of labeled glycine vs labeled ΔALA into heme (Table 3). Incorporation of ΔALA into heme was unchanged whereas glycine C\textsuperscript{14} incorporation was stimulated. These studies suggest that 5 β-H steroids induce ΔALA synthetase formation in human bone marrow cells.

These data thus extend to human erythroid cells the regulatory action of steroid metabolites on heme synthesis earlier demonstrated in avian liver and
embryonic red blood cell precursors by Granick and Kappas and their associates.5–7 We suggest that metabolites derived from androgenic steroids may play an important role in regulating the induction of hemoglobin synthesis and perhaps thereby regulating the differentiation of human stem cells into heme-synthesizing erythroid precursor cells.

ACKNOWLEDGMENTS

The authors thank Drs. A. Kappas and S. Granick of the Rockefeller University, New York, N.Y. for their invaluable advice and assistance. We wish also to thank Miss Elizabeth Stellato for her technical assistance.

REFERENCES

Studies on the Control of Hemoglobin Synthesis: The in vitro Stimulating Effect of a 5 β-H Steroid Metabolite on Heme Formation in Human Bone Marrow Cells

THOMAS F. NECHELES and UMA S. RAI