Stereospecific Tissue Uptake and Nuclear Accumulation of Testosterone in the Development of the Mouse Erythropoietic Spleen

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A double isotope ratio technique was used to estimate the specific binding of testosterone (T), as opposed to its biologically nonactive stereoisomer, epitestosterone (EpiT). The mouse erythropoietic spleen formed in response to a phenylhydrazine-induced hemolytic anemia was used as the target organ. Spleen minces from preanemic mice, as well as those in the early and late phases of erythropoietic spleen development, were incubated with $10^{-6}$ M of $^{14}$C-T and $^{3}$H-EpiT, and the selective uptake of T was calculated from the $^{14}$C/$^{3}$H ratio measured in the media before and after incubation, as well as in the subcellular fractions of the minces. Preferential uptake of T was seen in the early phase of development, but not in spleens obtained from preanemic animals or those in the late phase. There was no evidence of metabolic conversion of T or EpiT. The selective uptake of T by early phase spleens was reflected in a preferential nuclear accumulation of T. These data represent the first demonstration of a specific binding of T in vitro to a developing erythroid tissue.

There is ample evidence that testosterone (T)* and other androgens stimulate erythropoiesis in different species; however, the stimulation is a complex process which includes enhancement of the renal production of erythropoietin, synergistic effects of the steroid with erythropoietin, and a direct steroid action on erythroid precursor cell development. The evidence for the latter two sites of action includes the observation that direct addition of testosterone to human bone marrow cells results in a stimulation of DNA syn-
Byron reported that testosterone and 5 β-H androgen derivatives stimulated more mouse colony-forming cells (CFU) to enter the S phase of cell cycle than those obtained from animals untreated with steroids or exposed to 5 α-H androgens. The effect was apparently independent of the erythropoietin level of the donor mice. Finally, in studies utilizing 5 β-H androgen derivatives, a direct stimulatory effect was observed on heme and globin synthesis in bone marrow cultures of humans.

While the above studies attest to the biochemical effects of testosterone and other androgens on erythropoiesis, few data have been obtained on the mechanism of interaction between these hormones and the erythroid precursor cells. The molecular basis of steroid hormone action has recently been the subject of intensive study, and the first step in the chain of events was generally found to be a specific uptake of the hormone by its target tissue. The specific binding of the hormone must be distinguished from the nonspecific, as the latter may occur even at low concentrations of steroids in an incubation medium. In the present study, a novel approach was used to solve this problem. The specific tissue uptake and nuclear accumulation of T was compared to its biologically inactive stereoisomer, epitestosterone (EpiT), during the development of the mouse erythropoietic spleen following a phenylhydrazine-induced hemolytic anemia. The conversion from a predominantly lymphoid organ to an erythropoietic one is an orderly sequence of events of reproducible magnitude. This fact permits a division of the developmental chronology into an early phase, i.e., maximal rate of RNA synthesis, and a later phase, i.e., maximal rate of globin synthesis. The results of the present investigation indicate that a specific tissue uptake of testosterone occurred during the early phase of erythropoietic spleen (EEP) development but not during the preanemic or the late erythropoietic phases (LEP). There was no detectable metabolic transformation of T or EpiT following incubation with EEP splenic minces, a result consistent with systems utilizing rat bone marrow preparations.

MATERIALS AND METHODS

C57Bl/6J mice weighing 20-30 g were purchased from the Jackson Laboratories, Bar Harbor, Me. Sheep plasma erythropoietin (Step III, 2.2-3.2 units/mg protein) was purchased from Connaught Laboratories, Ltd., Willowdale, Ont., Canada. The radioisotopes, ¹⁴C-testosterone (58 mCi/mM), ³H-epitestosterone (55 Ci/mM), and ³H-testosterone (55 Ci/mM) were obtained from New England Nuclear Corporation, Boston, Mass. These were subjected to paper chromatography, eluted with ethanol, and stored with a concentration of 10⁵ cpm/ml at 4°C. The solutions were checked for purity every 3 mo and, if necessary, rechromatographed. ⁵⁹Fe as ferrous citrate (14.67 mCi/mg) was purchased from the Mallinckrodt Chemical Works, St. Louis, Mo. The nonradioactive steroids were purchased from Steraloids Inc., Pawling, N.Y., and stored in ethanol at 4°C. The organic solvents (chloroform, ethyl acetate, heptane, benzene, and methanol) were spectral grade. Adenosine, cytidine, deoxyctydine, guanosine, thymidine, and uridine from PL Laboratories, Milwaukee, Wis., were stored frozen at a concentration of 10⁻³ M. Eagle's minimum essential medium (MEM) was obtained from BBL, Inc., Cockeysville, Md. The thin-layer chromatography plates (Silica gel Ces F 254) were a product of Merck & Co., Rahway, N.J., and phenylhydrazine HCl was obtained from Fisher Scientific Co., Fair Lawn, N.J. The nonionic detergent Triton X-100 was supplied by J. T. Baker Chemical Co., Phillipsburg, N.Y., and the potassium polyvinyl sulfate (PVS) was obtained from Eastman Organic Chemicals, Rochester, N.Y.
Incubation of Spleen Minces

The mice received three intraperitoneal injections of neutralized phenylhydrazine (0.4 mg/10 g body weight). The injections were administered at 5:00 p.m. on day 1 as well as 9:00 a.m. and 5:00 p.m. on day 2. At selected time intervals following the initial injection, the mice were killed by cervical dislocation. The spleens were rapidly removed, placed on ice, and all the subsequent steps were performed at 4°C. The tissue was minced and separated into 1-g aliquots, which were washed with 8 ml of MEM. The suspension was centrifuged at 1500 g for 10 min, and the pellet was resuspended in a TKM-PVS buffer, except that KCl was omitted, following careful removal of debris from the sides of the centrifuge tubes. This suspension was centrifuged at 1500 g for 10 min. A 1-ml aliquot of the supernatant was kept for extraction and estimation of radioactivity, while 5 ml was transferred to polyallomer tubes for centrifugation in a SW 50.1 rotor (Beckman Instrument Co., Palo Alto, Calif.) at 100,000 g for 18 hr. Following centrifugation, a 1-ml aliquot was obtained from both the top and bottom halves of the samples to differentiate between the macromolecule-free (upper half) and the protein fraction (lower half). A control sample containing tissue-free medium was included in each experiment.

In the experiments in which the subcellular distribution of radioactivity was determined, the tissue minces were sedimented following incubation by centrifugation at 1500 g for 10 min. The minces were homogenized, using a motor driven Teflon pestle in four volumes of 0.25 M sucrose-TKM-PVS buffer (0.05 M Tris-Cl, pH 7.5; 0.025 M KCl; 0.005 M MgCl2; PVS, 10 μg/ml) and the homogenate was filtered through four layers of gauze. Two volumes of 2.3 M sucrose were mixed gently with the filtrate and 1½ volumes of 2 M sucrose were used to underlay the sample in a 50-ml polyethylene centrifuge tube. The tubes were then centrifuged at 50,000 g for 60 min and 10 ml of the supernatant was further centrifuged for 100,000 g for 60 min in a SW 40.1 rotor (Beckman Instrument Co.). This high-speed supernatant was kept and defined as the cytosol fraction. The initial 50,000 g pellet was suspended in 2 ml of 0.25 M sucrose-TM-PVS buffer (the same as TKM-PVS buffer, except that KCl was omitted), following careful removal of debris from the sides of the centrifuge tubes. This suspension was referred to as the nuclear pellet. At this point, Triton X-100 was added to the nuclear pellet in a final concentration (V/V) of 0.25% (protocol A) or 1% (protocol B). In both protocols, A and B, the detergent-treated nuclear pellet was agitated in a vortex and then centrifuged for 1500 g for 10 min; the Triton wash was retained for estimation of radioactivity. This fraction represented material from the outer and inner perinuclear membranes. The nuclear pellets were then washed with 2 ml of 0.25 M sucrose-TM-PVS buffer, and this fraction was centrifuged for the nuclear low salt extract. In protocol A, the pellets were then extracted with ethanol to obtain the residual nuclear extract. In protocol B, the pellet was washed with 0.4 M KCl-0.25 M sucrose-TM-PVS buffer, and the supernatant was designated as the nuclear high salt extract. The pellets were resuspended in the same high salt buffer, sonicated for 1 min at output setting 7 (Model 185D; Heat Systems, Ultrasomics, Inc., Plainview, N.Y.), and then centrifuged at 5000 g for 10 min. The supernatant was labeled nuclear residual fraction 1, and the pellet was extracted with 5 ml ethanol, and the supernatant, following centrifugation at 500 g for 10 min, was nuclear residual fraction 2.

Steroids were extracted from all aqueous fractions with 10 volumes of ethyl acetate and then with 10 volumes of chloroform using a vortex for mixing. The organic phases were pooled and evaporated to dryness in counting vials and 5 ml toluene-PPO-POPOP were added. Radioactivity determinations were done in a Mark II liquid scintillation counter (Nuclear Chicago, Arlington Heights, Ill.) with a double isotope setting of 52% efficiency for 3H and 78% for 14C. Each sample was assayed for up to 10,000 counts for a precision of 2%.
Study of Metabolism of T and EpiT by Spleen Minces

Spleen minces obtained from mice prior to administration of phenylhydrazine and 4 days following the initial dose were incubated for 30 min at 37°C with either \(^3\)H-T or \(^3\)H-EpiT (both had specific activities of 55 Ci/mM). The time of incubation was the same as that used in the determination of steroid uptake. The following fractions were analyzed: medium prior to and following incubation, cytosol, nuclear pellet, both 0.25%c, and 1%c Triton extracts, low and high salt extracts of nuclear pellet (protocol B), and ethanol extracts of nuclear pellet (protocol A and B). \(^1\)C-T was added to the samples containing \(^3\)H-T to determine the per cent of recovery. Since \(^1\)C-EpiT was not available, the percentage of recovery of \(^1\)C-T was used for the samples containing \(^3\)H-EpiT. After extraction as described above, 150 \(\mu\)g of nonradioactive T and EpiT were added to the appropriate samples.

Each sample was applied to a TLC plate and the chromatogram was developed in chloroform ether (190:10) for 45 min. This development was repeated twice in order to obtain better separation of the steroids. The plates were divided into six fractions: (1) the origin and polar compounds; (2) EpiT, T, etiocholanolone; (3) androsterone; (4) dihydrotestosterone; (5) androstenedione; (6) etiocholanolone and androstanolone. The \(R_F\) values of fractions 2-6 were 0.42, 0.52, 0.58, 0.68, and 0.79, respectively. Only in fraction 2 were significant amounts of radioactivity detected. This area was eluted with ethanol and transferred to Whatman paper No. 2 for descending chromatography with heptane:benzene:methanol:water (333:166:400:100) for 4 hr at 37°C. T, EpiT, and etiocholanolone were separated (\(R_F\) values were 0.41, 0.51, and 0.66, respectively), eluted with ethanol, and the radioactivity was determined.

Measurement of Plasma ESF Levels

Female Swiss Webster mice, 20-25 g, were kept for 2 wk in dimethyl silicone membrane enclosures (General Electric Corp., Schenectady, N.Y.). At 5 days following removal from the hypoxic environment, mice with hematocrits of 65%, or higher were used for the assays. The mice, in groups of four to five, were injected i.p. with erythropoietin in 0.1%c bovine serum albumin normal saline, or 0.8%c bovine serum albumin-normal saline, or saline dilutions of the plasma samples. The plasma was obtained from the C57Bl/6J mice by cardiac puncture, using a heparinized syringe, while the animals were anesthetized with ether. The plasma samples were incubated at 4°C for 6 hr with Swiss Webster mouse packed red cells, 200 \(\mu\)l cells/ml of plasma. This step precluded hemolysis in the recipient exhypoxic mice. The red cells were removed by centrifugation and 0.25 ml of plasma was injected into the polycythemic mice. After an interval of 48 hr, 1 \(\mu\)Ci of \(^5\)Fe\(^2+\) citrate was injected i.p. and, again, following an interval of 48 hr, 0.2 ml of blood was removed from the animals' orbital plexus for hematocrit and radioactivity determinations. Mice with hematocrits < 55%, were excluded from the assay.

Calculations

Data were processed through a computer program which included: (1) cpm of \(^1\)C-T; (2) cpm of \(^3\)H-EpiT; (3) \(^1\)C/\(^3\)H ratio. In addition, the \(^1\)C/\(^3\)H ratio of each sample (\(R_s\)) was compared to the \(^1\)C/\(^3\)H ratio of the medium prior to incubation (\(R_0\)) as follows:

\[
R = \frac{R_s - R_1}{R_0} \times 100.
\]

A minus sign was used to indicate a decrease of the ratio \(R\) and a plus sign, an increase of \(R\). Statistical analysis was done by use of the Student's two-tailed t test.

RESULTS

The experimental findings of this study are based on the determination of the \(^1\)C-T/\(^3\)H-EpiT ratio (\(R\)) remaining in the medium or present in subcellular fractions following incubation. T is the biologically active stereoisomer, while EpiT is devoid of androgenic, myogenic, or erythropoietic activity. When the
medium containing equimolar concentrations of $^{14}$C-T and $^3$H-EpiT comes into contact with a tissue which removes the steroids, $R$ may either remain the same or change: (1) If $R$ does not change—this indicates that there was not a stereospecific preference in the uptake of T or EpiT. (2) If $R$ decreases—this indicates a stereospecific uptake of T rather than EpiT. (3) If $R$ increases—this indicates a stereospecific uptake of EpiT rather than T.

Splenic minces were obtained from mice prior to as well as the fourth and seventh days following the induction of a hemolytic anemia with phenylhydrazine. These latter time points were coincident with the EEP and LEP. The minces were incubated with the labeled androgens, and the experimental results were analyzed as described above. A significant increase in uptake of T relative to EpiT was observed with day 4 splenic minces, but not those obtained prior to or 7 days after induction of the anemia (Fig. 1). The $R$ values for days 0 and 7 were $+3.5\pm 1.6$ and $-1.8\pm 1.4$, which were not statistically significant. In contrast the value of $R$ observed with day 4 splenic minces was $-10.1\pm 1.5$, this change being highly significant ($p < 0.001$). It was also different from the $R$ values of day 0 ($p < 0.001$) and day 7 ($p < 0.01$). This preference was exhibited at physiologic concentrations of the androgens, i.e., $1 \times 10^{-9} M$. The time of preferential uptake of T coincided with other early events of erythropoietic spleen development such as maximal production of cytoplasmic ribosomal RNA, peak nuclear RNA polymerase activities, maximal number of RNA containing nucleoli in splenic erythroblasts, maximal synthesis of splenic histones and nonhistone proteins, as well as the first detection of cytoplasmic globin mRNA activity following the onset of the anemia. In contrast, during the LEP when the organ was predominantly composed of hemoglobin containing erythroblasts, there was not a significant difference in the uptake of T or EpiT. The LEP is also the period of maximal globin chain synthesis as reported by Kazazian and co-workers.

In addition, there were significant differences in plasma ESF levels obtained from preanemic, EEP and LEP mice. The data are shown in Table 1. The percent of $^{59}$Fe injected recovered in erythrocytes of exhypoxic polycythemic
Table 1. ESF Levels in Phenylhydrazine-induced Hemolytic Anemia of C57BL/6J Mice*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Per Cent $^{59}$Fe Incorporation (mean ± SD)</th>
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<tbody>
<tr>
<td>Albumin-saline</td>
<td>2.24 ± 0.56</td>
</tr>
<tr>
<td>Plasma from day 0 mice</td>
<td>1.85 ± 1.07</td>
</tr>
<tr>
<td>Plasma from day 4 mice</td>
<td>17.88 ± 3.26</td>
</tr>
<tr>
<td>Plasma from day 7 mice</td>
<td>5.31 ± 2.57</td>
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</table>

* The relative ESF values of the donor mice plasma were assayed as described in Materials and Methods. Sheep plasma erythropoietin (Step III), 1.0 unit in 0.5 ml of 0.1% bovine albumin-saline, yielded a $^{59}$Fe incorporation of 9.7% ± 1.92.

Recipient mice was essentially the same when either albumin-saline or plasma from day 0 animals was injected. However, an approximately ninefold stimulation was observed following injection of plasma from day 4 mice and more than twofold with plasma from day 7 mice. The maximal ESF levels coincided with the nadir of the circulating hematocrit following induction of the anemia with phenylhydrazine.12

Factors, other than the stage of erythropoietic spleen development, were investigated for their effect on the relative uptake of T and EpiT. At each time point in spleen development, there was not a marked difference in the observed ratios correlative to the temperature of incubation (Table 2). In addition, no significant differences were observed between splenic minces obtained from male or female mice at days 0, 4, and 7 following administration of phenylhydrazine. The mean (± SD) changes in $R$ were as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Temperature (°C) of Incubation</th>
<th>Change of $R$ (mean ± SD)</th>
<th>No. of Experiments</th>
<th>Two-Tailed t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>−2.3 ± 5.5</td>
<td>5</td>
<td>$p &gt; 0.10$</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>+6.1 ± 6.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>−12.1 ± 5.9</td>
<td>6</td>
<td>$p &gt; 0.20$</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>−8.9 ± 6.1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>−2.7 ± 1.7</td>
<td>4</td>
<td>$p &gt; 0.20$</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>−1.2 ± 5.7</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

This finding was possibly related to the immaturity of the mice as animals above 30 g were excluded from the study. Also, it is known that steroids are adsorbed on the surfaces of various materials. In order to determine if this could affect the results, $^{14}$C/$^{3}$H ratios in the 1500 g supernatant, as well as the upper and lower halves of the 100,000 g supernatant, were compared to the ratios of the original media. The mean (± SD) changes in $R$ for the respective
fractions were $+1.9 \pm 5.9$ ($n = 11$), $+2.8 \pm 4.8$ ($n = 12$) and $+2.4 \pm 5.6$ ($n = 9$). This finding indicated that the adsorption of steroids on glass or polyallomer did not significantly alter the $^{14}C/^{3}H$ ratio. The above data indicated that, with the exception of the stage of erythropoietic spleen development, $R$ values were not altered by other variables which were studied.

**Absence of Metabolic Interconversion of T and EpiT by Splenic Minces**

As a stereospecific uptake of T was observed with day 4 splenic minces, it was of interest to determine if there was any evidence that T was converted to a derivative as is observed in the conversion to dihydrotestosterone prior to specific binding in the prostate. Minces, either from day 0 or day 4 animals, were incubated with $^{3}H$-T or $^{3}H$-EpiT and the following fractions were obtained: pre- and postincubation medium, cytosol, the Triton wash and the final nuclear extract (protocol A), the Triton wash, high salt wash, and final ethanol wash of the nuclear pellet (protocol B). The procedure of extraction as well as thin layer and paper chromatography are described in the Materials and Methods section. In all these experiments, only the original steroid was found in any of the fractions. Specifically, there was no transformation of T to EpiT, nor any conversion of these steroids to androstenedione, androsterone, etiocholanolone, or dihydrotestosterone. Furthermore, there was no evidence of significant formation of dihydroxysteroids. The recovery throughout this procedure was 90% or above, following organic extractions and at least 75% after thin layer chromatography.

**Evidence for Nuclear Accumulation of T During the Early Phase of Erythropoietic Spleen Development**

The $R$ values of subcellular fractions of splenic minces following incubation with $^{14}C$-T and $^{3}H$-EpiT were determined in specimens obtained at day 0 and day 4 following phenylhydrazine administration. In these experiments, as contrasted to the tissue uptake studies, a plus value for $R$ indicated stereospecific accumulation of T, rather than EpiT, while a minus value represented the opposite. The ratio of the cytosol fractions in these experiments were essentially unity.* However, analysis of the nuclear fractions prepared as described in the section on Materials and Methods demonstrated significant differences in the relative concentrations of T and EpiT. The nuclear pellets, prior to extraction, were essentially devoid of cytoplasmic contaminants after passage through sucrose. Following treatment with Triton X-100, the outer and inner perinuclear membranes were no longer present on the intact nuclei when viewed by electron microscopy (C. Kitchens and J. L. Spivak, unpublished observations). The $R$ values of the combined Triton plus low salt washes compared to the residual nuclear fractions at day 0 and day 4 are shown in Table 3. As demonstrated, the $R$ values on day 0 were negative, indicating a higher concentration of EpiT than T in the detergent and salt washes. The $R$ values of these washes

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*The $R$ values for the cytosol fractions were for day 0 (1.07, 1.09, 0.98) and day 4 (0.96, 1.03, 0.92).
Table 3. Per Cent Changes in $R$ of Subcellular Fractions of Preanemic and EEP Spleens

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Day 0 (n = 2)</th>
<th>Day 4 (n = 4)</th>
<th>Day 0 (n = 2)</th>
<th>Day 4 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton + low salt washes</td>
<td>-14.7%* (-8.7; -20.8)</td>
<td>+6.1% (+3.1 to +9.0)</td>
<td>-20.1% (−19.3; -20.9)</td>
<td>-19.9% (-12.2 to -25.6)</td>
</tr>
<tr>
<td>Residual nuclear fractions†</td>
<td>-4.0% (-3.8; -4.3)</td>
<td>+24.4% (+19.5 to 28.4)</td>
<td>-3.9% (-2.6; -5.3)</td>
<td>+24.1% (+21.7 to 27.1)</td>
</tr>
</tbody>
</table>

* Per cent change of $R$ value; mean (range).
† In protocol A, the residual fraction was the ethanol extract. In protocol B, the residual fraction included the high salt extract, nuclear residual fractions 1 and 2.

on day 4 were slightly positive, using protocol A and markedly negative when protocol B was employed. This difference may reflect the higher detergent concentration in the latter method. Using either protocols A or B, the extracts of the detergent-washed nuclear residues revealed a striking difference between samples obtained at day 0 and 4. The preanemic nuclear residues demonstrated a slight negative $R$ value, indicating either minimal accumulation of EpiT, rather than T or essentially equivalent uptake of the two steroids. In contrast, the day 4 nuclear residues were characterized by markedly positive values, mean $+24.4\%$ (protocol A) and mean $+24.1\%$ (protocol B). This finding indicated that, at physiologic concentrations, the splenic minces of the EEP preferentially accumulated T, rather than EpiT, in a nuclear-bound fraction and that such a preferential accumulation was not found in preanemic splenic minces.

**DISCUSSION**

The determination of the mechanism of steroid hormone action is complicated by the contribution of nonspecific binding that occurs in addition to the specific tissue interaction of the agent. In the present study, we have attempted to discriminate between the specific and nonspecific binding of T to an erythropoietic tissue by estimation of the $^{14}C/^{3}H$ ratio of T compared to its biologically inert stereoisomer, EpiT. The method, however, is indirect in two ways. (1) The estimation of tissue uptake of testosterone is measured by specific withdrawal from the medium rather than accumulation within the minces. This handicap is not pertinent to the experiments on nuclear accumulation. (2) The double isotope technique is a comparison of the relative concentrations of two enantiomers and, being restricted to that relationship, is an indirect method. The residual medium $R$ values following incubation with T and EpiT have been found to be independent of the temperature of incubation and the sex of the animals. In contrast, only splenic minces derived from mice in the EEP of spleen development have shown a stereospecific uptake of T. This finding represents the first demonstration of a growth-phase specific (early as opposed to late phase of erythropoietic development) interaction of T with an erythropoietic tissue. Minguell and Grant have reported the in vivo uptake of T in rat bone marrow where the distinction between early and late erythroid cell precursors is not as pronounced as in the spleen at different stages of development. These authors also have demonstrated that there is little, if any, evidence for the
metabolic conversion of T to other androgens such as dihydrotestosterone. A similar result is seen in our work using the mouse spleen system, with essentially no observable conversion of T or EpiT to either 5 \( \alpha \)-H or 5 \( \beta \)-H derivatives. These data suggest that, at least in recognizable stages of erythroid development, i.e., proerythroblasts, etc., any direct effect of T would be as the parent molecule and not as a metabolic derivative formed in the developing erythroblast. Finally, the heightened uptake of T is reflected as an accumulation in a nuclear fraction separate from the outer and inner perinuclear membranes. Although biologic function cannot be predicated from subcellular localization, the relative enrichment for T in nuclei resembles the events in more well-defined examples of steroid cell interaction.

When translated into actual concentrations, the specific uptake of T at day 4 is \( 3.4 \pm 1.9 \times 10^{-12} \ M/g \) wet weight tissue, and the nuclear accumulation represents \( 3.6 \times 10^6 \) molecules/\( \mu g \) nuclear DNA or 20–30 molecules/mouse diploid nucleus. These values may represent a minimal estimate of the specific androgen-binding capacity of the target cell for the following reasons: (1) A decrease may occur in binding capacity under in vitro, rather than in vivo, conditions. (2) T may not represent androgen with the highest binding efficiency to receptor molecules of the early phase spleen. The direct effects of 5 \( \beta \)-H steroids, rather than T, on heme and globin synthesis of bone marrow cells of mouse and man, and the effects of anterythropoietin on the in vivo actions indicate that the 5 \( \beta \)-H steroids have a direct effect on some stage of the developing erythroblast. The present experiments may indicate that T has a low, but sufficient, affinity for cellular receptor sites at which other androgens, i.e., 5 \( \beta \)-H steroid derivatives, bind with a higher affinity. (3) T may bind to cellular receptor sites with the same affinity as other androgens, but the cellular concentration of such receptors may be low as a function of the stage of maturity of the developing erythroid precursor. At day 4 following phenylhydrazine, the dominant cells by morphologic and biochemical criteria are pro- and basophilic erythroblasts. However, evidence obtained in whole animal studies suggests that the direct effect of androgens occurs early in development on the CFU or a stage of development immediately succeeding the commitment of CFU to erythroid precursors. The specific uptake of T in the present study may reflect a vestigial concentration of cell receptors which decay during the maturation of the CFU to a proerythroblast. There is the possibility that the observed tissue uptake of T is not by the splenic erythroblasts but some other cellular element, e.g., component(s) of the hematopoietic microenvironment. At this point, it is not feasible to distinguish the cell types which may be involved as autoradiography is prohibited by the very small specific uptake of T. (4) The selective disappearance of T from the medium, as well as its accumulation in the residual nuclear fractions, may reflect a change in the transport of one or both of the labeled steroids.

During the course of the present study, Valladares and Minguell reported the characterization of a protein isolated from rat bone marrow cell nuclei which can specifically bind T and, as inferred from competition experiments, 5 \( \alpha \) and 5 \( \beta \) dihydrotestosterone to a lesser extent. However, there is uncertainty as to the biologic function of this receptor, as the authors report that
there has been no indication of T binding by cytosol receptors. This finding may be a reflection of the paucity of early precursor forms in a resting bone marrow cell population.

REFERENCES

Sterospecific tissue uptake and nuclear accumulation of testosterone in the development of the mouse erythropoietic spleen

AJ Hadjian, JL Spivak, A Kowarski, HW Dickerman and CJ Migeon