Extrahepatic Metabolism of Progesterone in Pseudopregnant Rats

IDENTIFICATION OF REDUCTION PRODUCTS*

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Previous investigations reported from this laboratory have shown that progesterone-4-C\textsuperscript{14} administered intravenously to female rats deprived of liver, spleen, and gastrointestinal tract was converted into at least six metabolic products (1). The major product, zone 2, isolated from carcasses of these animals, was identified as 20a-hydroxypreg-4-en-3-one (2). Three compounds were separated from zone 1 by repeated chromatography but were not completely characterized (1). This report describes the identification of the remaining metabolites comprising zones 3, 4, and 5 and the distribution of labeled steroids in uterine tissues of eviscerated pseudopregnant rats.

EXPERIMENTAL PROCEDURE

Materials—All solvents, of analytical grade, were redistilled prior to use. Reagents, also of analytical grade, were used as obtained from the suppliers. Oxidized and reduced triphosphopyridine nucleotide were purchased from Sigma Chemical Company. Purity of reference steroids was assessed through melting point determination and paper chromatography (see Table I). Chromatographically homogenous progesterone-4-C\textsuperscript{14} (20 µg per pmole) obtained from Nuclear-Chicago was dissolved in ethanol (5 µg/0.05 ml) and added, with thorough mixing, to undiluted rat plasma to a concentration of 5 µg/5.0 ml. A standard dose of 0.09 µg/100 g of body weight was injected into each animal.

Animals—Young female Holtzman rats, 90 to 120 days old, were fed Staley's Rockland rat diet and water ad libitum. Animals were housed in quarters with regular periods of light and darkness fixed automatically. Vaginal smears were taken daily, and only those animals exhibiting regular 5-day cycles were used. Rats were made pseudopregnant by electrical stimulation of the cervix on the day of proestrus. Deciduomas were induced by scratching the antimesometrial aspect of the uterine lumen with a barbed needle. Rats were functionally eviscerated by a modification of the Russell technique (3). In this operation, the abdominal viscera, except the liver, are removed, and the liver is left in situ with its entering circulation cut off. The animal so prepared is in most respects equivalent to one that has been completely eviscerated. Groups of pseudopregnant rats (6 animals) in which one horn of the uterus had been traumatized to provoke decidual growth were eviscerated at different times (15 minutes, and 1, 2, 4, and 5 days) after trauma. Immediately after evisceration, each animal was given an injection of progesterone-4-C\textsuperscript{14} into the saphenous vein. After 1 hour, animals again were anesthetized and killed by exsanguination through the abdominal aorta.

Assay of C\textsuperscript{14} in Blood and Tissues—The methods used are the same as previously reported (1).

Isolation of Labeled Steroids—Blood and tissue homogenates diluted with water were extracted five times with equal volumes of ethyl acetate. After evaporation of solvent under nitrogen, the lipid residue was dissolved in 70% methanol at 40°. The methanol solutions were kept at -15° for 10 minutes. The pellets were rinsed with cold 70% methanol, and the rinsings were added to their corresponding supernatant fractions. Methanol solutions were reduced in volume under a stream of nitrogen at 40° to 3 ml and diluted to 15 ml with water. Labeled material was quantitatively removed from these aqueous solutions by ethyl acetate extractions. After evaporation of solvent, the labeled material was chromatographed in hexane-formamide on 2- or 3-cm strips of Whatman No. 1 filter paper. Labeled components were measured directly on the paper chromatograms by scanning with a thin window flow counter and automatic recording device. Zones of radioactivity were eluted from the strips with methanol in an elution vessel described by Zander and Simmer (4).

Detection and Measurement of Ultraviolet Absorption—The Haines scanner (5) was used for the detection of ultraviolet-absorbing material on the paper chromatograms. For more accurate determination of absorption maxima and quantitative determination of material absorbing maximally at 240 µµ, methanol solutions of eluted steroids were read in a Cary model 11 recording spectrophotometer.

Color Reactions—Reference and carrier steroids were routinely located on chromatograms by the iodine reaction (6). Steroids insensitive to this reagent were detected with more specific reagents: 5α-pregnane-3,20-dione by means of the Zimmermann reaction (7), 3α-hydroxy-5α-pregn-20-one with antimony trichloride (8), and the various pregnanediols with phosphomolybdic acid (9).

Acetylation of Steroids The steroid, dissolved in 0.4 ml of pyridine and 0.25 ml of acetic anhydride, was allowed to stand at room temperature overnight, and extracted from the reaction mixture with hexane after the addition of 3 ml of water. Partial

* This investigation was supported in part by Grant A.756 from the National Institutes of Health.
Acetylation of steroid samples was achieved with 0.45 ml of pyridine and 0.05 ml of acetic anhydride.\(^1\)

**Chronic Acid Oxidation of Steroids**—Freshly prepared 0.4% \(\text{Cr}_2\text{O}_3\) in 90% acetic acid was added to the dry steroid sample, and the vessel containing the mixture was stoppered and set aside at room temperature for 1 hour. After the addition of 3 ml of water, the steroid sample was extracted with hexane.

**Enzymic Oxidation-Reduction**—The steroid was dissolved in 0.1 ml of ethanol; 3.0 ml of phosphate buffer, 0.1 \(\text{m}\), \(\text{pH} 8.5, 0.009\) unit of purified rat ovary \(20\alpha\)-hydroxysteroid dehydrogenase (10), and 0.5 \(\mu\) mole of triphosphopyridine nucleotide were added. The mixture was incubated at 37° for 1 hour. Quantitative and specific oxidation of the \(20\alpha\)-hydroxyl group resulted when less than 5 \(\mu\)g of steroid were used. Reduction of \(20\beta\)-ketosteroids to \(20\alpha\)-hydroxysteroids by the reverse reaction required 3.0 ml of 0.1 \(\text{m}\) phosphate buffer at \(\text{pH} 6.0\), and 0.5 \(\mu\) mole of reduced triphosphopyridine nucleotide. Steroids were extracted from the reaction mixtures with ethyl acetate.

**Formation of Girard's T Complex** (11)—To the dry steroid was added 0.1 ml of a solution of 100 mg of trimethylacethydrazide ammonium chloride dissolved in 0.5 ml of acetic acid. The glass-stoppered sample was allowed to stand at room temperature overnight. Ice-water, 3 ml, was added, and the solution was neutralized to \(\text{pH} 7.0\) with 10% \(\text{NaOH}\). Nonketonic steroid components were extracted three times with 20 ml of peroxide-free ether. The Girard's T-ketonic steroid complex was dissociated by the addition of 4% \(\text{H}_2\text{SO}_4\) and 2 hours standing at room temperature. Ketonic steroids were extracted from the acidified solution with ether.

**Formation of 2,4-Dinitrophenylhydrazones**—Method A-1 of Reich, Crane, and Sanfilippo (12) was used as described, except that smaller volumes of reagents were employed.

**Sodium Borohydride Reduction**—\(\text{NaBH}_4\), 0.25 mg, was added to the steroid sample dissolved in 0.25 ml of cold methanol, and the mixture was kept at 0° for 1 hour. After the addition of 1 ml of water, the steroid was extracted with ethyl acetate.

**Diaphragm Precipitation**—Twenty milligrams of 3\(\beta\)-hydroxy-5\(\beta\)-pregnane-3,20-dione and 2000 c.p.m. of Compound 5 were dissolved in 2.0 ml of \(0.95\%\) ethanol, and 3 ml of \(0.5\%\) ethanolic digitonin solution (1%) were added. Precipitation required approximately 30 minutes. After 18 hours at room temperature, the precipitate was filtered and washed with cold ethanol and ether. After the complex was dissolved in pyridine and water was added, the carrier steroid was extracted with ether.

**Saponification of Steroid Acetates**—Steroid acetates were saponified in a solution of 1 ml of ethanol and 0.2 ml of 4% aqueous \(\text{NaOH}\) at reflux temperature for 1 hour. After addition of water, the steroids were extracted with ethyl acetate.

**Carrier Analyses**—Labeled metabolites were admixed with 25 \(\mu\)g of reference steroid and chromatographed. The chromatograms were scanned for radioactivity, and the reference steroids were located either with the Haines scanner or by color test. Carrier analysis by recrystallization employed a variety of solvents, and acetate derivatives were formed when possible. Specific activities of crystals and mother liquor were determined by plating 1 to 2-ml aliquots on aluminum disks and counting in a windowless gas flow counter. Replicate plates, usually four, were prepared from each sample to give a measure of the combined plating and weighing errors.

### RESULTS

**Tissue Distribution of \(\text{C}^{14}\)**

Distribution of labeled steroids in the tissues 1 hour after injection was essentially the same as previously reported (1); however, lower values were obtained in the present study (Table II). Concentration of \(\text{C}^{14}\) in liver tissue was generally lower than that in blood of the same animal, which contrasts with previous observations in normal animals, in which uptake by liver was rapid (1). Administration of labeled progesterone to animals in which the left uterine horn had been traumatized 5 days before resulted in a higher concentration of \(\text{C}^{14}\) in the horn containing the deciduoma than in the control horn (Table II). Variation of the distribution of \(\text{C}^{14}\) into the two uterine horns as a function of time after trauma is given in Fig. 1. While no change was observed in the control horns in which no tissue growth occurred, the traumatized horns showed a sigmoidal increase in \(\text{C}^{14}\) concentration that paralleled the increase in uterine weight. In none of the other tissues analyzed was any significant variation of \(\text{C}^{14}\) distribution observed during pseudopregnancy.

**Chromatography of Tissue Extracts**

Ethyl acetate extraction of tissue homogenates and diluted blood quantitatively removed all labeled material except in the case of the liver where only approximately 60% of the isotope was extractable. Removal of nonlabeled lipid contaminants by cold 70% methanol was performed without loss of labeled material. Therefore, except for liver, chromatography of the

### TABLE I

**Physical properties of reference steroids**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Melting point (°C)</th>
<th>Chromatographic mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td>Progesterone</td>
<td>121-123</td>
<td>0.38</td>
</tr>
<tr>
<td>5(\beta)-Pregnane 3,20-dione</td>
<td>120-121</td>
<td>0.65</td>
</tr>
<tr>
<td>5(\alpha)-Pregnane 3,20-dione</td>
<td>203-205</td>
<td>0.65</td>
</tr>
<tr>
<td>3(\alpha)-Hydroxy-5(\beta)-pregnan-20-one</td>
<td>140-150</td>
<td>0.44</td>
</tr>
<tr>
<td>3(\beta)-Hydroxy-5(\beta)-pregnan-20-one</td>
<td>142-143</td>
<td>0.55</td>
</tr>
<tr>
<td>3(\alpha)-Hydroxy-5(\beta)-pregnan-20-one</td>
<td>194-195</td>
<td>0.44</td>
</tr>
<tr>
<td>20(\alpha)-Hydroxy-5(\beta)-pregnan-3-one</td>
<td>178-180</td>
<td>0.39</td>
</tr>
<tr>
<td>20(\alpha)-Hydroxy-5(\beta)-pregnan-3-one</td>
<td>237-239</td>
<td>0.43</td>
</tr>
<tr>
<td>3(\delta),20(\beta)-Dihydroxy-5(\beta)-pregnan-20-one</td>
<td>227-232</td>
<td>0.55</td>
</tr>
<tr>
<td>3(\alpha),20(\beta)-Dihydroxy-5(\beta)-pregnan-20-one</td>
<td>242-243</td>
<td>0.27</td>
</tr>
<tr>
<td>3(\alpha),20(\beta)-Dihydroxy-5(\beta)-pregnan-20-one</td>
<td>217-218</td>
<td>0.28</td>
</tr>
<tr>
<td>5(\alpha)-Pregnane-3,20-dione</td>
<td>120-121</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* These systems were used at 37°; all others were used at room temperature. All melting points were determined on a Kofler hot stage and are corrected.

\(^1\) Appreciation is expressed to Dr. Oscar Dominguez for details of this method, supplied prior to its publication.
TABLE II

Distribution of C\textsuperscript{14} 1 hour after progesterone-4-C\textsuperscript{14} administration to eviscerated rats bearing uterine deciduomas

Pseudopregnant rats were eviscerated 5 days after traumatization of the left uterine horn, treated by injection with progesterone-4-C\textsuperscript{14}, and killed 1 hour after injection. Values given are averages from 6 animals with the standard deviations.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight (mg)</th>
<th>Specific activity (c.p.m./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5200 ± 30</td>
<td>12.1 ± 0.1</td>
</tr>
<tr>
<td>Uterus, control horn</td>
<td>145 ± 3</td>
<td>9.0 ± 1.2</td>
</tr>
<tr>
<td>Uterus, traumatized horn</td>
<td>1298 ± 450</td>
<td>18.3 ± 2.6</td>
</tr>
<tr>
<td>Ovaries</td>
<td>90 ± 0</td>
<td>20.0 ± 1.2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1697 ± 22</td>
<td>24.9 ± 0.6</td>
</tr>
<tr>
<td>Adrenals</td>
<td>60 ± 0</td>
<td>26.6 ± 1.3</td>
</tr>
<tr>
<td>Liver</td>
<td>9970 ± 550</td>
<td>11.9 ± 2.7</td>
</tr>
</tbody>
</table>

extracts represented the total labeled steroid content of the tissue. Since all tissue extracts contained qualitatively the same pattern of metabolites as that observed in blood, which duplicated the earlier study (1), only the results from blood and decidual tissue are presented (Fig. 2). The qualitative resemblance of the isotope patterns in the two tissues is apparent. Relative amounts of different metabolites varied from tissue to tissue due presumably to variations in enzyme activities within the tissues. Chromatography of extracts from control and traumatized uterine horns, however, showed that the relative amounts of the metabolites remained constant in uterine tissue regardless of the degree of decidual tissue formation and the total quantity of C\textsuperscript{14} present in the tissues.

Characterization of Metabolites

Zone 5—Rechromatography of zone 5 in hexane-formamide resulted in its separation into two components. The least polar, \(R_F 0.65\), was designated Compound 5a, and the more polar, \(R_F 0.55\), was designated Compound 5. Neither compound absorbed ultraviolet light. The two could be more completely separated by acetylation followed by rechromatography. Compound 5a was unaffected, while Compound 5 was acetylated.

Compound 5a was resistant to CrO\textsubscript{4} oxidation and reacted with the ketonic reagents, 2,4-dinitrophenylhydrazine and Girard’s T. It chromatographed with the same mobility as both 5\(\alpha\)- and 5\(\beta\)-pregnane-3,20-dione. Identification of Compound 5a as the \(5\alpha\) epimer was established by carrier analysis. Pertinent data are given in Table III.

Oxidation of Compound 5 with CrO\textsubscript{4} yielded 5\(\alpha\)-pregnane-3,20-dione. The metabolite was not oxidized by 20\(\alpha\)-hydroxy-steroid dehydrogenase; however, a product with the same chromatographic mobility as zone 3 was formed by enzymatic reduction. Both 3\(\beta\)-hydroxy-5\(\alpha\)- and 3\(\alpha\)-hydroxy-5\(\beta\)-pregnane-20-ones chromatographed with Compound 5. Precipitation of the 3\(\beta\)-hydroxy-5\(\beta\) compound with digitonin from a mixture con-

FIG. 1. Pseudopregnant rats were eviscerated at intervals from 15 minutes to 5 days after traumatization of the left uterine horn, immediately treated by injection with progesterone-4-C\textsuperscript{14}, and killed 1 hour after injection. The concentration of C\textsuperscript{14} per unit (dry weight) of uterine tissue was determined for both traumatized (O—O) and control (●—●) horns. Each point represents the average of 6 determinations; standard deviations are indicated by subtended lines.

FIG. 2. Chromatography in hexane-formamide of extracts of blood (top) and traumatized uterus (bottom). Both tissues were taken 1 hour after progesterone-4-C\textsuperscript{14} injection into a pseudopregnant, eviscerated rat in which the left uterine horn had been traumatized 5 days previously. Zones of radioactivity are indicated by numbers 1 to 5.

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TABLE III

Carrier analysis by recrystallization

<table>
<thead>
<tr>
<th>Metabolic product</th>
<th>Carrier added</th>
<th>Free compound</th>
<th>Acetate derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>First</td>
</tr>
<tr>
<td></td>
<td></td>
<td>crystallization</td>
<td>crystallization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.p.m./mg.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>Compound 5a</td>
<td>5β-Pregnane-3,20-dione</td>
<td>72.2 ± 3.0</td>
<td>77.8 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a</td>
<td>5α-Pregnane-3,20-dione</td>
<td>26.9 ± 2.3</td>
<td>24.2 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 5</td>
<td>3β-Hydroxy-5α-pregnan-20-one</td>
<td>1310</td>
<td>415&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 5</td>
<td>3β-Hydroxy-5α-pregnan-20-one</td>
<td>215.0</td>
<td>198.9&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 4</td>
<td>Progesterone</td>
<td>157.4 ± 13.5</td>
<td>147.8 ± 11.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a</td>
<td>20α-Hydroxy-5α-pregnan-3-one</td>
<td>419.0</td>
<td>96 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a</td>
<td>20α-Hydroxy-5α-pregnan-3-one</td>
<td>32.5 ± 2.9</td>
<td>29.3 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a</td>
<td>20α-Hydroxy-5α-pregnan-3-one</td>
<td>108.4 ± 9.9</td>
<td>93.8 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a (oxidized)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5β-Pregnane-3,20-dione</td>
<td>189.9</td>
<td>124.8 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 3a (oxidized)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3β-Pregnane-3,20-dione</td>
<td>146.3 ± 13.0</td>
<td>102.0 ± 18.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3β-Pregnane-3,20-dione</td>
<td>1310</td>
<td>415&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Heptane.
<sup>b</sup> Methanol.
<sup>c</sup> The figures in parentheses refer to the specific activities of the mother liquor.
<sup>d</sup> Ethyl acetate.
<sup>e</sup> Acetone.
<sup>f</sup> Ethanol.
<sup>g</sup> Hexane-acetone (2:1).
<sup>h</sup> Hexane.
<sup>i</sup> Stepwise oxidation proceeding first with 20α-hydroxysteroid dehydrogenase followed by CrO₃.

The recrystallization analysis confirmed the identification of Compound 5 as 3α-hydroxy-5α-pregnan-20-one as shown in Table III.

Zone 4—This zone chromatographed like progesterone, and sufficiently large samples absorbed ultraviolet light maximally at 240 mμ. However, acetylation of the peak followed by rechromatography produced a second component. The material that acetylated and did not absorb ultraviolet light was designated Compound 3a. It reacted positively with ketonic reagents and was oxidized either by CrO₃ or by 20α-hydroxysteroid dehydrogenase, yielding, by both methods, 5α-pregnan-3,20-dione. Its chromatographic mobility matched that of 20α-hydroxy-5α-pregnan-3-one and 20α-hydroxy-5α-pregnan-3-one. Carrier crystallization confirmed its identification as the latter compound (see Table III).

That portion of zone 4 which did not acetylate was identified by carrier analysis as progesterone, as shown also in Table III.

Zone 5—Preliminary studies indicated that this region consisted of a single steroid (R<sub>f</sub> 0.27 in hexane-formamide), which did not absorb ultraviolet light. It was unreactive with ketonic reagents and sodium borohydride. CrO₃ oxidation produced 5α-pregnan-3,20-dione, and oxidation with 20α-hydroxysteroid dehydrogenase yielded 3α-hydroxy-5α-pregnan-20-one. Partial acetylation of Compound 3 gave a mixture composed mainly of monoaacetates with lesser amounts of diacetate and unreacted starting material. Oxidation of the monoaacetate fraction with CrO₃ followed by saponification and chromatography gave a mixture consisting of 20α-hydroxy-5α-pregnan-3-one (72%) and 3α-hydroxy-5α-pregnan-20-one (28%). Sodium borohydride reduction of 20α-hydroxy-5α-pregnan-3-one yielded a single product more polar than Compound 3, with the same mobility as 3β,20α-dihydroxy-5α-pregnan. Similar reduction of the 3α,20α-dihydroxy-5α-pregnan-20-one gave a mixture of two compounds, 10% chromatographing like Compound 3 and 90% being less polar and of the same mobility as 3α,20β-dihydroxy-5α-pregnan. Identification of Compound 3 as 3α,20α-dihydroxy-5α-pregnan was provided by carrier analysis as shown in Table III.
Identification of Progesterone Metabolites

DISCUSSION

A summary of the enzymatic reduction of progesterone in the eviscerated female rat is presented in Fig. 3. Taylor (13) has identified 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnane-20-one as metabolites of progesterone in female rat liver preparations. Other work (14) has shown that livers from female rats reduce ring A of Δ4-3-ketosteroids at faster rates than those from males, and that this sex difference results from a greater concentration of the microsomal Δ4-5α-hydrogenase in the female. The present study establishes that the Δ4-5α-hydrogenase and the 3α- and 20α-hydroxysteroid dehydrogenase activities have been measured in ovaries, kidneys, muscle, and peritoneal fat from mature female rats and that this sex difference results from a greater concentration of the microsomal Δ4-5α-hydrogenase in the female. The presence of any metabolites with β orientation in female extrahepatic tissues is ruled out by the complete absence of additional peaks of radioactivity in initial or subsequent chromatograms of tissue extracts.

This study does not permit a detailed discussion of the distribution of enzymes among various extrahepatic tissues; however, 20α-hydroxysteroid dehydrogenase activity has been measured in ovaries, kidneys, muscle, and peritoneal fat from mature female rats but was not measurable in uterine tissue, and Δ4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase activities have been observed in rat ovaries, kidneys, and uteri (15).

The absence of highly polar metabolites of progesterone-4-C14 from the eviscerated rat preparation has been reported (1). In this study, radioactivity was quantitatively extracted from all tissues, except liver, and prepared for chromatography without significant loss. Therefore, characterization of the isolated steroid components accounts for at least 80% of the total metabolites by extrahepatic tissues. The 40% of labeled material unextractable from liver probably represents highly polar steroid conjugates synthesized in the liver. The absence of these polar components from other tissues provides additional evidence of the functional inertness of the liver in this animal preparation.

Of critical importance to this investigation was the proper identification of Compound 5α and the CrO3 oxidation products of Compounds 3, 3α, and 5, since this characterization would fix the configuration of the entire series as either αα or αβ. Because both 5α- and 5β-pregnane-3,20-diones have identical mobilities in all chromatographic systems tested in this laboratory (see also Savard (16)), other methods of distinction were necessary. Melting point is an excellent differentiation when sufficient quantities of material are available, but was of no help with micro amounts. Carrier crystalization analysis provided the only method applicable at the time; however, this technique had to be carried out with care in the selection of solvents, as constant ratio crystalization did occur between 5α pregnane 3,20-dione-4-C14 and carrier 5β-pregnane-3,20-dione (Table III). As a consequence of this difficulty, it was erroneously reported (15) that CrO3 oxidation of Compounds 3 and 5 yielded 5β-pregnane-3,20-dione. I wish to acknowledge this error and to retract the earlier report. The assigned configuration as αα is established without doubt by an extension of the recrystallization analysis. Correlative evidence, provided through the identification of all metabolites in the series, makes an error in the designation of the proper A to B ring junction highly improbable.

Assignment of the 20α-hydroxy configuration to Compounds 3 and 3α is firmly established through the use of the 20α-hydroxy-steroid dehydrogenase the specificity of which has been reported in some detail (10). Contaminating levels of 3α-hydroxysteroid dehydrogenase in crude preparations of rat ovary 20α hydroxy-4-dehydrogenase have been observed (15); consequently, adequate purification necessarily preceded its use in characterization studies.

In addition to the carrier crystalization studies in which direct comparison with authentic compounds was made, another line of evidence supported the designation of 3α-hydroxy configuration to Compounds 3 and 5. Three compounds displayed chromatographic mobilities less polar than their 3β counterparts, in harmony with the fact previously noted by Savard (16) that compounds with axial hydroxyl configuration, being more hindered and less reactive, also are chromatographically less polar. Under conditions that acetylated only part of the available hydroxy groups of Compound 3, the 20α-hydroxyl group reacted with greater ease than that at position 3, suggesting that the latter group was more hindered. Reduction of the 3-keto group of an A:B-trans steroid, i.e. 20α-hydroxy-5α-pregnan-3-one, at neutral pH, yields the unhihdered, equatorial hydroxyl product, 3β,20α-dihydroxy-5α-pregnane (17). This pregnanediol was more polar than Compound 3.

Lower concentrations of labeled steroids in rat blood and organs in the present study compared with those previously obtained (1) requires comment. The difference undoubtedly arises from use of the Russell evisceration technique which does not involve interference with the general venous circulation. The Ingle preparation (18), on the other hand, requires ligation of the vena cava. Restriction of blood circulation in this manner would hinder penetration of labeled steroids into extremities, resulting in a higher concentration of C14 in the blood and those organs with a better blood supply.

In consideration of the possible correlation of this study with the metabolism of endogenous progesterone in the normal rat, both progesterone and 20α-hydroxyprogren-4-en-3-one have been isolated in approximately equal amounts from ovaries, peritoneal

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2 W. G. Wiest, unpublished observations.

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Recent experience in this laboratory has indicated that vapor phase chromatography may provide a convenient method of distinction even with micro quantities.
fat, and blood of pregnant rats (19). This observation harmonizes with the observation of 20α-hydroxysteroid dehydrogenase activity in rat ovaries (10, 15) and with the finding of 20α-hydroxyprog-4-en-3-one as the major metabolite in eviscerated rats (2).

The formation of deciduomas in the embryo-free uterus is generally regarded as tantamount to the development of normal maternal placental tissue (20). In rats, this reaction requires proper hormonal conditioning of the uterus, the production of the progravid endometrium, which occurs during pseudopregnancy. Genesis of the deciduoma seems to depend on a two-stage process, i.e. the initiatory stage, requiring an inflammatory stimulation of the endometrium, and the proliferative stage which begins between 36 and 48 hours after stimulation and is characterized by rapid increase in number and size of stromal cells. In both stages, progesterone plays a necessary role (20).

The present study does not provide information on the role of progesterone in the initiatory process. However, the increased capacity of uterine tissue to retain labeled steroids, beginning approximately 48 hours after trauma (Fig. 1), bears a striking similarity in time of response to the increased rate of P32 incorporation into nucleic acids of deciduomal tissue observed by Gold and Sturgis (21) and to the polyploidy and increased rate of mitotic activity measured by Sachs and Shelesnyak (22). The requirement for progesterone in the development of decidua requires a demand for the hormone by the growing tissue which could be met by the increasing ability of the tissue to retain the hormone.

**SUMMARY**

Carrier experiments employing paper chromatography, formation of chemical derivatives, and recrystallization to constant specific activity have been successfully used to identify 5α-pregnan-3, 20-dione, 3α-hydroxy-5α-pregnane-20-one, 20α-hydroxy-5α-pregnan-3-one, and 3α, 20α-dihydroxy-5α-pregnane as metabolic products of progesterone in the eviscerated female rat. Δ4-5α-Hydrogenase and 3α- and 20α-hydroxysteroid dehydrogenase activities are therefore implicated in extrahepatic tissues of this animal. An increasing capacity of uterine decidua tissue to retain administered progesterone was observed, and it was suggested that this increased retention might play a role in support of the decidual reaction.

A previous report from this laboratory assigning 5β configuration to three of the aforementioned metabolites is corrected in this presentation.

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