NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microimage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.
ANDROGEN GLUCURONIDES:
MARKERS OF IN VIVO AND IN VITRO
ANDROGEN METABOLISM

by

David L. Thompson, BSc(Hon.), MSc.

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
at
Dalhousie University
Halifax, Nova Scotia
May, 1990

C Copyright by David Lester Thompson, 1990
The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.
DEDICATION

To those family members and friends who patiently waited.
# TABLE OF CONTENTS

| TITLE PAGE | ........................................ | i |
| CERTIFICATE OF EXAMINATION | ........................................ | ii |
| COPYRIGHT AGREEMENT FORM | ........................................ | iii |
| DEDICATION | ........................................ | iv |
| TABLE OF CONTENTS | ........................................ | v |
| LIST OF TABLES | ........................................ | xi |
| LIST OF FIGURES | ........................................ | xii |
| ABSTRACT | ........................................ | xiii |
| LIST OF ABBREVIATIONS | ........................................ | xiv |
| ACKNOWLEDGEMENTS | ........................................ | xv |
| PREAMBLE | ........................................ | 1 |
| REVIEW OF LITERATURE | ........................................ | 4 |
| I. ANDROGENS AND THEIR ACTIONS | ........................................ | 8 |
| A/ THE ANDROGENS | ........................................ | 8 |
| B/ THE ENZYMES | ........................................ | 9 |
| i. 5α-REDUCTASE | ........................................ | 12 |
| ii. GLUCURONYL TRANSFERASE | ........................................ | 13 |
| C/ EFFECTS OF ANDROGENS ON SKIN | ........................................ | 15 |
## II. STEROID PRODUCING TISSUES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/ OVARY</td>
<td>17</td>
</tr>
<tr>
<td>B/ TESTIS</td>
<td>20</td>
</tr>
<tr>
<td>C/ ADRENAL</td>
<td>21</td>
</tr>
<tr>
<td>D/ SUMMARY</td>
<td>23</td>
</tr>
</tbody>
</table>

## III. PATHWAYS OF ANDROGEN METABOLISM

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/ ANDROGEN PRECURSORS</td>
<td>25</td>
</tr>
<tr>
<td>i. ANDROSTENEDIONE</td>
<td>25</td>
</tr>
<tr>
<td>ii. DEHYDROEPIANDROSTERONE</td>
<td>31</td>
</tr>
<tr>
<td>iii. DEHYDROEPIANDROSTERONE SULFATE</td>
<td>3</td>
</tr>
<tr>
<td>B/ POTENT ANDROGENS</td>
<td>36</td>
</tr>
<tr>
<td>i. TESTOSTERONE</td>
<td>36</td>
</tr>
<tr>
<td>ii. DIHYDROTESTOSTERONE</td>
<td>45</td>
</tr>
<tr>
<td>C/ UNCONJUGATED ANDROGEN METABOLITES</td>
<td>49</td>
</tr>
<tr>
<td>i. ANDROSTANEDIOL</td>
<td>50</td>
</tr>
<tr>
<td>ii. ANDROSTERONE</td>
<td>53</td>
</tr>
<tr>
<td>D/ ANDROGEN GLUCURONIDES</td>
<td>55</td>
</tr>
<tr>
<td>i. TESTOSTERONE GLUCURONIDE</td>
<td>56</td>
</tr>
<tr>
<td>ii. DIHYDROTESTOSTERONE GLUCURONIDE</td>
<td>59</td>
</tr>
<tr>
<td>iii. ANDROSTANEDIOL GLUCURONIDE</td>
<td>61</td>
</tr>
<tr>
<td>vi. ANDROSTERONE GLUCURONIDE</td>
<td>65</td>
</tr>
</tbody>
</table>

## IV. SUMMARY | 67 |
<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Part A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Adiol-G and Adiol 3-G levels in serum of normal men and in normal men infused with $^3$H-dihydrotestosterone</td>
<td>104</td>
</tr>
<tr>
<td>Introduction</td>
<td>105</td>
</tr>
<tr>
<td>Methods</td>
<td>105</td>
</tr>
<tr>
<td>Results</td>
<td>106</td>
</tr>
<tr>
<td>Summary</td>
<td>110</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Part B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiol 3-G and Adiol 17-G levels in serum of normal men</td>
<td>111</td>
</tr>
<tr>
<td>Introduction</td>
<td>112</td>
</tr>
<tr>
<td>Methods</td>
<td>112</td>
</tr>
<tr>
<td>Results</td>
<td>113</td>
</tr>
<tr>
<td>Summary</td>
<td>115</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Part A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production of Adiol-G by glucuronyl transferase prepared from dispersed rat prostate cells</td>
<td>116</td>
</tr>
<tr>
<td>Introduction</td>
<td>117</td>
</tr>
<tr>
<td>Methods</td>
<td>118</td>
</tr>
<tr>
<td>Results</td>
<td>120</td>
</tr>
<tr>
<td>Summary</td>
<td>124</td>
</tr>
<tr>
<td>EXPERIMENT 2</td>
<td>PART B</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>PRODUCTION OF ADIOL-G BY GLUCURONYL TRANSFERASE PREPARED FROM HOMOGENIZED RAT LIVER</td>
<td>125</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>126</td>
</tr>
<tr>
<td>METHODS</td>
<td>126</td>
</tr>
<tr>
<td>RESULTS</td>
<td>128</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>132</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EXPERIMENT 2</th>
<th>PART C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRODUCTION OF ADIOL-G BY GLUCURONYL TRANSFERASE PREPARED FROM HOMOGENIZED HUMAN LIVER</td>
<td>134</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>135</td>
</tr>
<tr>
<td>METHODS</td>
<td>135</td>
</tr>
<tr>
<td>RESULTS</td>
<td>138</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>138</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V. EXPERIMENT 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANDROSTERONE GLUCURONIDE: A MARKER OF ADRENAL HYPERANDROGENISM</td>
</tr>
<tr>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>METHODS</td>
</tr>
<tr>
<td>RESULTS</td>
</tr>
<tr>
<td>SUMMARY</td>
</tr>
</tbody>
</table>

| VI. SUMMARY | 158 |

ix
<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PERCENT CROSS-REACTION OF ANTISERUM AGAINST 5α-ANDROSTANE-3α,17β-DIOL 3-GLUCURONIDE-BSA</td>
</tr>
<tr>
<td>2</td>
<td>ANDROGEN CONJUGATE RETENTION TIMES ON HPLC USING 35:65 ACETONITRILE:WATER AS ELUTING SOLVENT</td>
</tr>
<tr>
<td>3</td>
<td>PERCENT CROSS-REACTION OF ANTISERUM AGAINST 5α-ANDROSTANE-3α,17β-DIOL 17-GLUCURONIDE-BSA</td>
</tr>
<tr>
<td>4</td>
<td>ANDROGEN CONJUGATE RETENTION TIMES ON HPLC USING 40:60 ACETONITRILE:WATER AS ELUTING SOLVENT</td>
</tr>
<tr>
<td>5</td>
<td>PERCENTAGE OF $^3$H-ANDROSTANEDIOL GLUCURONIDE PRESENT AS $^3$H-ANDROSTANEDIOL 3-GLUCURONIDE IN SERUM OF NORMAL MEN FOLLOWING INFUSION OF [9,11]-$^3$H-DIHYDROTESTOSTERONE</td>
</tr>
<tr>
<td>6</td>
<td>SERUM LEVELS OF ANDROSTANEDIOL 3-GLUCURONIDE, ANDROSTANEDIOL 17-GLUCURONIDE AND TOTAL ANDROSTANEDIOL-GLUCURONIDE IN NORMAL MEN</td>
</tr>
<tr>
<td>7</td>
<td>PERCENTAGE OF $^3$H-ANDROSTANEDIOL GLUCURONIDE PRESENT AS $^3$H-ANDROSTANEDIOL 3-GLUCURONIDE FOLLOWING INCUBATION OF RAT PROSTATE GLUCURONYL TRANSFERASE WITH $^3$H-ANDROSTANEDIOL</td>
</tr>
<tr>
<td>8</td>
<td>ANDROSTANEDIOL 3-GLUCURONIDE AND TOTAL ANDROSTANEDIOL-GLUCURONIDE LEVELS IN RAT SERUM</td>
</tr>
<tr>
<td>9</td>
<td>AGES, HIRSUTISM SCORES, BODY-MASS INDICES AND NUMBER OF SUBJECTS PER GROUP IN ANDROSTERONE-GLUCURONIDE STUDY</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PATHWAYS OF STEROID METABOLISM</td>
</tr>
<tr>
<td>2</td>
<td>HPLC ELUTION PROFILE OF ANDROSTANEDIOL 3-GLUCURONIDE</td>
</tr>
<tr>
<td>3</td>
<td>HPLC ELUTION PROFILE OF ANDROSTANEDIOL 17-GLUCURONIDE</td>
</tr>
<tr>
<td>4</td>
<td>SEPARATION OF UNCONJUGATED TESTOSTERONE AND ANDROSTANEDIOL BY CELITE CHROMATOGRAPHY</td>
</tr>
<tr>
<td>5</td>
<td>HPLC ELUTION PROFILE OF ANDROSTERONE-GLUCURONIDE</td>
</tr>
<tr>
<td>6</td>
<td>HPLC ELUTION PROFILE OF RAT PROSTATE GLUCURONYL TRANSFERASE 3H-PRODUCTS: COMPARISON WITH 3H-ANDROSTANEDIOL 3-GLUCURONIDE AND 3H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES</td>
</tr>
<tr>
<td>7</td>
<td>HPLC ELUTION PROFILE OF RAT HEPATIC GLUCURONYL TRANSFERASE 3H-PRODUCTS: COMPARISON WITH 3H-ANDROSTANEDIOL 3-GLUCURONIDE AND 3H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES</td>
</tr>
<tr>
<td>8</td>
<td>HPLC ELUTION PROFILE OF HUMAN HEPATIC GLUCURONYL TRANSFERASE 3H-PRODUCTS: COMPARISON WITH 3H-ANDROSTANEDIOL 3-GLUCURONIDE AND 3H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES</td>
</tr>
<tr>
<td>9</td>
<td>ANDROSTANEDIOL-GLUCURONIDE ISOMER PRODUCTION FROM ANDROSTANEDIOL BY HUMAN HEPATIC GLUCURONYL TRANSFERASE AS DETERMINED BY RADIOIMMUNOASSAY</td>
</tr>
<tr>
<td>10</td>
<td>BASELINE ANDROGEN GLUCURONIDE LEVELS IN NORMAL, OBESE AND HIRSUTE WOMEN</td>
</tr>
<tr>
<td>11</td>
<td>SERUM ANDROSTERONE-GLUCURONIDE LEVELS FOLLOWING EITHER OVARIAN SUPPRESSION OR COMBINED OVARIAN-ADRENAL SUPPRESSION</td>
</tr>
<tr>
<td>12</td>
<td>SERUM ANDROSTERONE-GLUCURONIDE LEVELS BEFORE AND 60 MINUTES AFTER INTRA-VENOUS ACTH ADMINISTRATION IN NORMAL AND HIRSUTE WOMEN</td>
</tr>
</tbody>
</table>
ABSTRACT

Androgens stimulate the metabolic activity of androgen sensitive tissues. One consequence of unabated androgen stimulation is abnormal tissue growth. Androgen metabolism within androgen sensitive tissues is therefore an important mechanism for the maintenance of normal tissue development. The terminal step in androgen metabolism is conjugation of the androgens to either glucuronide or sulfate moieties. This thesis examines androgen glucuronide production as a measure of androgen metabolism.

Specific radioimmunoassays for four androgen glucuronides have been developed: Androstanediol 3-glucuronide (Adiol 3-G); androstanediol 17-glucuronide (Adiol 17-G); total androstanediol glucuronide (Adiol-G); and androsterone glucuronide (Andros-G). In addition, a glucuronyl transferase assay has been developed.

The data presented show that androgens are metabolized to androgen glucuronides by both peripheral and hepatic tissues. These results question the validity of using serum androgen glucuronide levels as markers specific for peripheral androgen metabolism.

Pathways of dihydrotestosterone (DHT) metabolism are described. The data presented show that Adiol 17-G is the major Adiol-G isomer in serum. Additional data suggest that this Adiol 17-G is formed by conversion of DHT to Adiol followed by selective glucuronidation of Adiol's 17-carbon hydroxyl.

By measuring the changes in Andros-G and/or Adiol-G levels following changes in ovarian and/or adrenal steroidogenesis, it has been determined that adrenal steroidogenesis is the principle source of elevated androgen-glucuronide levels normally seen in hirsute women.

Androgen glucuronides are therefore useful indicators of adrenal steroid metabolism by both the liver and peripheral tissues.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>ADIOL</td>
<td>Androstenediol</td>
</tr>
<tr>
<td>ADIOL 3-G</td>
<td>Androstenediol 3-glucuronide</td>
</tr>
<tr>
<td>ADIOL 17-G</td>
<td>Androstenediol 17-glucuronide</td>
</tr>
<tr>
<td>ANDROS-G</td>
<td>Androsterone-glucuronide</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone-sulfate</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DHT-G</td>
<td>Dihydrotestosterone-glucuronide</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
</tr>
<tr>
<td>IH</td>
<td>Idiopathic hirsutism</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>PCO</td>
<td>Polycystic ovarian syndrome</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to thank the Department of Physiology and Biophysics for their patience in the preparation of this manuscript.

For the use of his personal computer, I would like to thank Mr. Michael Schedlich.

For their invaluable assistance in developing the glucuronyl transferase assays, for their laughter and for their understanding, I would like to thank Ms. C. Leopold and Ms. H. Zwicker.

I would like to thank Dr. W. Moger and Dr. C. Lazier for their invaluable assistance as members of my advisory committee. In addition, I am deeply indebted to Dr. Moger for the years of patient guidance shown during my years in his laboratory. I respect Dr. Moger as a learned scholar, I value his thoughtful and provocative council and I admire his interesting outlook on life.

My years spent working with Dr. R. Rittmaster have, without a doubt, been the most productive and rewarding years of my scientific career. Roger has taught me a great deal about science and about myself. I leave his lab, taking with me many skills I did not possess before meeting him. I will miss the time that we have spent together.

I know many people, but refer to only a few as "friend". Hugh James Wickham is my friend. On many occasions, Hugh convinced me to carry on and finish what I started, even though I felt I was incapable of doing so. I thank Hugh for the strength he gave me when I needed it most.

Lynn and I came to Nova Scotia by ourselves. We leave with three beautiful children. To my wife and children, I say thank you for your unqualified love, your remarkable patience and, last but not least, your humour. You have made my load lighter by being there.
Androgens are steroid hormones, responsible for sexual differentiation in the fetus, development of secondary sexual characteristics in the pubescent male and maintenance of spermatogenesis in the adult male. In addition, they serve as substrate for the production of estrogens in the female. The metabolism of androgens is complex, producing in some cases an amplification of, and in other cases a dampening of, the androgenic signal.

Termination of the signal is accomplished by excretion of the androgens, or their metabolites. Such excretion occurs primarily through the urine. Since androgens are steroids, they are not readily soluble in urine, making excretion difficult. Conjugation of the steroid nucleus to either a glucuronide or sulfate moiety renders the steroid more water soluble and therefore more easily filtered into the urine.

5α-Androstane-3α,17β-diol glucuronide (androstanediol glucuronide - Adiol-G) is an androgen metabolite. Currently it is thought that this conjugate is produced from 5α-androstane-17β-ol,3-one (dihydrotestosterone - DHT) metabolism within peripheral tissues; the splanchnic compartment is not thought to contribute to the circulating pool of Adiol-G. This suggests that serum levels of Adiol-G would be an excellent marker of androgen stimulation within peripheral androgen-sensitive tissues.
Many reports have shown that hyperandrogenic women presenting with excessive hair growth (hirsutism) have elevated Adiol-G levels. The increased hair growth results from elevated levels of DHT acting within the skin to stimulate hair follicle development. The elevated Adiol-G levels in serum are believed to result from the metabolism of this DHT by skin.

The data presented in this thesis question whether androgen glucuronide formation is, in fact, a useful marker of peripheral DHT metabolism. Three issues are addressed:

1) is Adiol-G produced from the metabolism of DHT by peripheral tissues only?

2) is the glucuronidation step the initial step in terminating the DHT signal, or is DHT first metabolized to 5α-androstane-3α,17β-diol (Adiol) and the resulting Adiol then glucuronidated?

3) do the adrenal and ovary both contribute equally to the androgens which are ultimately metabolized to androgen glucuronides?

To determine if serum androgen glucuronide levels are a measure of peripheral androgen metabolism only, this thesis compares androgen glucuronide formation by the hepatic and
extrahepatic compartments in both rat and human. The in vitro conversion of Adiol, DHT and 5α-androstane-3α-ol,17β-one (androsterone) to androgen glucuronides by rat prostate, rat liver and human liver is reported. These results are compared to the levels of androgen glucuronides present in serum. Based on these comparisons, inferences are drawn regarding the contribution of the two compartments to the overall production of the conjugates.

The pathway of DHT conversion to androgen glucuronides is examined by determining whether the glucuronyl transferase enzymes prepared from rat prostate and rat liver exhibit substrate specificity.

Finally, the contribution of ovarian and adrenal steroidogenesis to the production of a second androgen conjugate, androsterone glucuronide (Andros-G) is examined in normal and hirsute women.

From these data, a model linking DHT metabolism to androgen glucuronide formation is presented.
REVIEW OF LITERATURE
DHT stimulation of androgen dependent peripheral tissues is critical to the normal functioning of these tissues. Should DHT production be increased, or DHT elimination be decreased, hyperstimulation of these tissues would result. Such hyperstimulation could produce undesirable conditions such as benign prostatic hyperplasia in men or hirsutism in women. Clinical treatment of these conditions could be initiated earlier in their development if a highly selective marker for peripheral DHT metabolism could be identified. Such early treatment would undoubtedly produce less suffering for the patient. Therefore, efforts have been made to identify a serum marker of peripheral DHT metabolism which is elevated above the normal range in all hyperandrogenic subjects. This thesis examines the utility of androgen glucuronide formation as a marker of peripheral DHT metabolism.

The literature review summarizes the currently available information on the production and elimination of DHT. This is done by dividing the literature review into four sections:

SECTION I. This is an introductory section, presenting basic background information on the steroids, enzymes and physiological parameters dealt with in the remaining three sections of the literature review.
The steroids which contribute directly or indirectly to the production or metabolism of DHT will be introduced. These steroids are categorized as either: 1) androgen precursors; 2) androgens; 3) unconjugated androgen metabolites; or 4) glucuronidated androgen metabolites.

The information presented in the literature review will establish that androgen glucuronides appear to be the best currently detectable serum markers of peripheral DHT metabolism. For this reason, data describing the enzyme glucuronyl transferase will be reviewed since this enzyme is responsible for glucuronidation of unconjugated steroids. A second enzyme, 5α-reductase, will also be discussed since this enzyme is responsible for the production of DHT from 4-androstene-17β-ol-3-one (testosterone). This step is a critical step in the expression of androgenic activity within peripheral tissues.

Elevated DHT metabolism within skin results in a stimulation of hair growth. Measuring the degree of hair growth in regions of skin not normally covered by pigmented hair is one method of assessing androgenization in females. Since this method of assessment is used in many of the reports presented in the literature review as well as in Experiment 3 of this paper, the actions of androgens on skin are also reviewed.
SECTION II. Very little DHT is actually secreted by steroidogenic tissues. Rather, DHT is produced within target tissues from the metabolism of other steroids. Therefore, to study the production of DHT, one must first study the production of these other steroids. For this reason, the second section of the literature review describes the control of ovarian, testicular and adrenal steroidogenesis.

SECTION III. In this section, the pathways leading to the formation and elimination of DHT will be reviewed. The pathways of elimination include those leading to the production of unconjugated androgen metabolites as well as androgen glucuronides.

SECTION IV. This section is a summary of the data presented in the review of literature.
I.

ANDROGENS AND THEIR ACTIONS

A/ THE ANDROGENS

The steroids to be discussed in this section are divided into four categories based upon their chemistry and biologic activity: 1) androgen precursors; 2) androgens; 3) unconjugated androgen metabolites and 4) conjugated androgen metabolites.

The androgen precursors include 4-androsten-3,17-dione (androstenedione), 5-androsten-3β-ol-17-one (DHEA), 5α-androstane-3,17-dione, and 5-androsten-3β-ol-17-one-3-sulfate (DHEAS). These steroids contribute to overall androgenicity by serving as substrate for the production of active androgens. They themselves do not bind to the androgen receptor and as such do not have intrinsic androgenic activity (Geller 1975).

The androgens include testosterone and its 5α-reduced metabolite DHT. Both testosterone and DHT stimulate androgen-sensitive tissues, but their relative efficacy varies. In some tissues, such as skin and prostate, testosterone’s effects are expressed only after conversion
to DHT (Bruchovsky 1968). This irreversible conversion is catalyzed by the enzyme 5α reductase.

The unconjugated androgen metabolites include Adiol and androsterone. These steroids are produced in part from the metabolism of testosterone and DHT.

Conjugation of an androgen or its metabolite to either a glucuronide or sulfate moiety results in increased excretion of that steroid in urine. Androstanediol glucuronide (Adiol-G) and androsterone glucuronide (Andros-G) are the predominant 5α-reduced androgen glucuronides present in serum.

B/ THE ENZYMES

The metabolic pathways of interconversion between the different androgens are shown in Figure 1. 5α reductase and glucuronyl transferase are two important enzymes in the production and termination of the androgenic signal. The activity and distribution of each enzyme will be briefly reviewed.
PATHWAYS OF ANDROGEN METABOLISM

Steroid Abbreviations:
- Testosterone-G: Testosterone glucuronide
- DHT: Dihydrotestosterone
- DHT-G: Dihydrotestosterone glucuronide
- Adiol: Androstanediol
- Adiol 3-G: Androstanediol 3-glucuronide
- Adiol 17-G: Androstanediol 17-glucuronide
- Andros: Androsterone
- Andros-G: Androsterone glucuronide

Enzyme Key:
1. Glucuronyl Transferase
2. 17-keto steroid reductase
   or
   17-hydroxy steroid dehydrogenase
3. 5α-reductase
4. 3-keto steroid reductase
   or
   3-hydroxy steroid dehydrogenase
FIGURE 1
i. 5α Reductase

DHT is produced from testosterone by the enzyme 5α-reductase. Andersson et al (1989) determined by DNA sequence analysis, that 5α-reductase in rat liver and rat prostate are identical proteins, having a molecular weight of 29 kDa. They also showed that expression of 5α-reductase activity is tissue dependent. Castration resulted in elevated production of the mRNA in rat liver but not rat prostate. In contrast, testosterone treatment in castrate rats induced prostatic but not liver mRNA levels for this enzyme.

Mauvais-Jarvis et al (1981) have also shown differential expression of 5α-reductase between tissues. They showed that hepatic 5α-reductase activity in hypothyroid men was diminished while skin 5α-reductase activity remained normal.

Expression of 5α-reductase activity also varies between different regions of skin. Prior to puberty, male and female genital skin (scrotal and labial) have high 5α-reductase activity while pubic skin 5α-reductase activity is very low (Mauvais-Jarvis et al 1983).

Cultured skin fibroblasts have been shown to exhibit significant 5α-reductase activity (Wilson 1975). Not all fibroblasts displayed the same degree of activity however.
Fibroblasts from genital skin had greater 5α-reductase activity than did fibroblasts from pubic skin.

These data show that expression of 5α-reductase activity is tissue specific. As such, the control over the production of DHT is also tissue specific.

ii. Glucuronyl Transferase

The production of androgen glucuronides requires the conjugation of glucuronic acid to a steroid nucleus by the enzyme glucuronyl transferase. The tissue distribution and substrate specificity of this enzyme determine which metabolic intermediates in the metabolism of DHT are produced and by which tissues. For these reasons, the distribution and substrate affinities of glucuronyl transferase will be reviewed.

The conjugation of a steroid to glucuronic acid by the enzyme glucuronyl transferase occurs at an unconjugated hydroxy group on the steroid nucleus. The glucuronide moiety is ‘donated’ by uridine diphospho-glucuronic acid (UDPGA).

Many diverse tissues have been shown to possess glucuronyl transferase activity. These include human liver (Irshaid and Tephly 1987), rat liver (Matsui and Nagai 1985), human lung (Milewich et al 1985), human breast (Raju et al 1983) and rat kidney (Coughtrie et al 1987).
Unlike 5α-reductase, there is more than one glucuronyl transferase. Matsui and Nagai (1985) purified hepatic glucuronyl transferase from male Wistar rats. They found that there were two different forms of this enzyme, one form glucuronidated androsterone, while the second form glucuronidated 4-nitrophenol. Since there is a Wistar rat subpopulation having very low liver 3α-hydroxy steroid glucuronyl transferase activity, they compared the enzyme profiles from rats with normal activity to those from rats with low activity. They found that there were two isoenzymes in the normal rat which displayed 3α-hydroxy steroid glucuronyl transferase activity, and that one of these isoenzymes is missing in the low-activity rat model. In another report, Matsui and Nagai (1986) purified an hepatic glucuronyl transferase from rat liver which was specific for the 17β-hydroxyl group. This glucuronyl transferase accepted testosterone as substrate.

Radomsinska et al (1986) showed that the 3-hydroxy steroid glucuronyl transferase of rat liver microsomes glucuronidated many different substrates, but that there was a preferential glucuronidation of 3α- over 3β-hydroxyl groups.

Therefore, there are multiple forms of glucuronyl transferase, and they are present in many different tissues. The androgen-glucuronides produced within a given tissue from the metabolism of a given steroid will then depend upon
the form of glucuronyl transferase present within that tissue.

C/ EFFECTS OF ANDROGENS ON SKIN

Androgen glucuronide levels are elevated in hirsute women. In order to better understand the basis behind this relationship, the factors governing hair growth will be briefly reviewed.

One of the more pronounced changes initiated during puberty is the growth of hair in the pubic and axillary regions. In men, beard growth is also stimulated. These changes are the result of androgens acting upon hair follicles within these regions.

Except for the palms and soles, hair follicles are present over the entire surface of the body. Hair is generally described as either vellus or terminal. While vellus hair is short, fine and unpigmented, terminal hair is coarse, long and contains pigment in various amounts. In androgen dependent skin, androgens (and to a smaller degree estrogens) convert vellus hair to terminal hair (Rittmaster 1987). The only regions of normally hairy skin on which androgens have no effect are the corona of the scalp, eyelashes and eyelids; other regions require androgen stimulation for terminal hair formation.
Hyperandrogenism in women can result in an increased conversion of vellus to terminal hair in regions not normally covered by terminal hair, such as the face, upper abdomen and back. This condition is known as hirsutism and is commonly used as a clinical marker of female hyperandrogenism (Rittmaster 1987).

The DHT which stimulates hair growth appears to be produced within the skin itself. While terminal hair follicles have modest 5α-reductase activity, sebaceous glands located near the base of the hair shaft have been shown to have significant 5α-reductase activity (Dijkstra 1987).

In conclusion, 5α-reductase activity in skin stimulates terminal hair growth by increasing the formation of, and therefore the stimulation by, DHT. If androgen levels rise high enough, vellus hair will turn to terminal hair. Women who undergo dramatic changes in hair growth are referred to as hirsute. This is a commonly used measure of androgenicity in women and is used as such in Experiment 3 of this thesis.
II.
STEROID PRODUCING TISSUES

This section summarizes those factors controlling ovarian, testicular and adrenal steroidogenesis. This information is included since these control systems ultimately regulate the production of DHT and as well as androgen-glucuronides.

In women, the steroids present in highest concentration which contribute to androgen production are androstenedione, DHEA and DHEAS (Osborn and Yannone 1971). In men, testosterone is the major circulating androgen.

The production of these steroids will now be reviewed.

A/ OVARY

In primary follicles (follicles in the earliest stages of development), granulosa cells form a thin covering over the ovum. As the follicle develops, there is a proliferation of the granulosa cell layer. Not all granulosa cells within the granulosa cell layer appear to have the same steroidogenic capabilities. The density of luteinizing hormone (LH) receptors and content of certain steroidogenic enzymes are greatest near the follicular basal lamina (Amsterdam et al 1975). Immediately exterior to the basal lamina are the theca interstitial cells. The theca
cells are generally placed into two categories, theca interna and theca externa. A second class of interstitial cell, termed secondary interstitial or stromal cells, exist throughout the cortex and medulla of the ovary. These cells are thought to be theca interna cells from atretic follicles. They undergo hypertrophy and can respond to LH (Erickson et al. 1985).

Androgen production by the ovary occurs in the thecal and stromal cell compartment. These cells have been shown to produce primarily androstenedione, testosterone (Barbieri et al. 1986) and androsterone (Erickson et al. 1985). Ovarian androgen production is stimulated by luteinizing hormone (LH) (Magoffin and Erickson 1982).

Ovarian secretion of testosterone and androstenedione is elevated late in the follicular phase of the menstrual cycle as a consequence of rising LH levels (McNatty 1976). The intra-ovarian source of this elevated androgen production is not clear. Hodgen (1982) suggested that the dominant follicle is the primary source while Aedo et al. (1980 a,b) reported that the stromal compartment is the site of production.

Androstenedione production by the ovary varies from a minimum of 3.5 μmol/day to a maximum of 10.5 μmol/day depending on the day of the cycle (Longcope 1986). This accounts for 50 to 75% of total androstenedione production (the remainder coming from the adrenal). The ovary produces
50% of the secreted testosterone (Genazzani 1977) but total secretion (ovary + adrenal) only accounts for one half of circulating testosterone, the remaining testosterone arises from peripheral metabolism of androstenedione.

Increased ovarian androgen production can result in a variety of disorders, from irregular or anovulatory menstrual periods to outright virilization. Polycystic ovarian disease (PCO) is a disorder normally associated with hyperandrogenism (Mahesh and Greenblatt 1964), though a small percentage of women presenting with PCO have testosterone levels within the normal range (DeVane et al 1975). PCO women do not ovulate regularly, are heavier than age matched controls and have a greater probability of being hirsute. In addition, PCO is characterized by an increase in circulating LH levels (Yen 1980). This elevated LH results in hyperstimulation of the stromal cell compartment of the ovary, which in turn results in over production of androstenedione and testosterone.

In summary, the ovary produces primarily androstenedione and testosterone. This production is under the control of LH. Hyperstimulation of ovarian androgen production can result in virilization.
B/ TESTIS

Testicular steroidogenesis occurs within the Leydig (interstitial) cell and Sertoli (sust antic ular) cell compartments.

Androstenedione, DHEA, DHEAS, DHT, progesterone and 17 $\alpha$-hydroxy progesterone have been detected in the testis. However, the major steroid produced by the testis is testosterone. Testosterone is produced almost exclusively within the Leydig cell compartment.

Leydig cell production of testosterone is stimulated by LH (Shikita and Hall 1967; Dufau et al 1971). This production is amplified by follicle stimulating hormone (FSH) (Purvis and Hanson 1978) and prolactin (Hafiez et al 1972).

DHT can be produced within the testis since Sertoli cells display 5$\alpha$-reductase activity. However, DHT does not appear to be secreted from the testis in significant amounts.

Testicular hyperandrogenism is rare but can occur from LH secreting tumours. Leydig cell tumours are rare and usually benign.

In summary, the major steroid produced within the testis is testosterone. This production is stimulated by LH.
The adrenal gland is composed of two embryologically distinct regions, the outer cortex and inner medulla. Adrenal steroidogenesis occurs in the cortical layer.

The adrenal cortex is composed of three histologically distinct zones: zona glomerulosa, zona fasciculata and zona reticularis. Adrenal androgen production occurs in the zona fasciculata and zona reticularis regions (Jefcoate 1986). These two regions make up 70% of the adrenal cortex. The zona fasciculata is arranged in radial columns extending from the zona glomerulosa (outer layer of cortex) to the zona reticularis (inner cortical zone). Blood flow through the adrenal is unique in that it enters through the adrenal capsule and travels through each of the cortical layers in turn. This results in perfusion of the deeper cortical regions by blood which has already taken up secretions from the outer layers. Whether this pattern of blood flow exerts local control over the steroidogenic activities of the deeper cortical regions is not clear.

The major androgens produced by the adrenal cortex are androstenedione, DHEA and DHEAS (Weiland 1965). In addition, there is a small but significant production of testosterone (Baird 1969).
Rosenfeld et al (1975) showed that adrenal secretion of DHEA is episodic being temporally linked to the secretion of cortisol. This suggests that adrenocorticotropin (ACTH), the major stimulus for adrenal cortisol secretion, is also the major stimulus for adrenal androgen production. This control system has been examined further. Adrenal vein infusion of ACTH in normal women resulted in significant elevation of DHEA (Nieschlag et al 1973), androstenedione (Weil 1979) and testosterone (Givens et al 1975). However, adrenal androgen production increases at adrenarche (when DHEAS levels rise, age 6-7 years) while ACTH and cortisol levels do not. This has led to the hypothesis that there is a second factor, also thought to be from the pituitary, controlling adrenal androgen synthesis. A glycopeptide has been isolated from human pituitary which can deferentially stimulate canine adrenal cells to release DHEA but not cortisol (Parker et al 1983).

Pathologic changes in adrenal androgen production can cause virilization in the female. The most common conditions associated with altered adrenal androgen production include Cushing’s syndrome and congenital adrenal hyperplasia (CAH).

The hyperandrogenism associated with Cushing’s syndrome results from either over production of adrenal steroids by adrenocortical tumours or over stimulation of the adrenals by ACTH producing tumours.
CAH is the result of enzymatic defects within otherwise normal adrenocortical tissue. The enzyme most commonly implicated is 21-hydroxylase. A decrease in the activity of this enzyme results in lowered cortisol production. This in turn results in elevated ACTH release from the pituitary. The adrenal cortex undergoes hyperplasia in response to the elevated ACTH levels thereby returning cortisol production towards normal. However, the production of adrenal androgens is also increased by both the enzyme block and increased ACTH stimulation. This hyperandrogenism, if severe, can cause virilization in females.

In summary, the adrenal produces androstenedione, DHEA and DHEAS in response to ACTH. Other possible control systems may exist, but these have not yet been adequately characterized.

**D/ SUMMARY**

Gonadal steroidogenesis and adrenal steroidogenesis are controlled independently. Gonadal steroidogenesis is stimulated by LH while adrenal steroidogenesis is stimulated by ACTH.

Altered androgen production by the gonad or adrenal could result in hyperandrogenism. Such alterations could result from tumours within these steroidogenic tissues or their over stimulation by the appropriate pituitary factors.
III. PATHWAYS OF ANDROGEN METABOLISM

While DHT is the most potent androgen within many androgen sensitive tissues, very little DHT is actually produced and secreted by the gonad or adrenal. Rather, DHT is produced within androgen sensitive tissues from the metabolism of testosterone or other androgen precursors / metabolites.

Elevated tissue DHT levels result from either increased conversion of precursors or decreased metabolism of DHT to androgen-conjugates. Such elevated tissue DHT levels would result in hyperstimulation of tissue metabolism. Various pathological conditions result from such hyperstimulation. To better understand how such hyperstimulation occurs, the normal pathways of DHT production and elimination within such tissues must be reviewed.

Many of the steroids examined in this section are produced by both the gonad and adrenal. Increased steroidogenesis within either of these glands could result in elevated DHT production within the periphery. Proper management of such hyperandrogenism then depends upon identifying which gland is the principal source of steroid over production. Data addressing this issue in idiopathic hirsute and PCO women will be presented where available.
A/ ANDROGEN PRECURSORS

The steroids androstenedione, DHEA and DHEAS do not have any intrinsic androgenic activity since they do not bind to the androgen receptor. However, they can be metabolized to testosterone and DHT. For this reason they will be referred to as androgen precursors.

i. Androstenedione

To determine whether androstenedione is an androgen precursor, Pearlman and Pearlman (1961) infused androstenedione into male rats and measured the metabolites produced within various tissues. They found that polar compounds (possibly androgen conjugates) and 5β-DHT were the major products made by liver, while ventral prostate produced DHT, androstanedione and testosterone. The ratio of conjugated to free steroid was highest in the liver. In the human, prostate tissue has been shown to metabolize androstenedione to androstanedione, androsterone and epiaandrosterone (5β-androsterone) (Harper et al 1974). Testosterone and DHT were also produced, but at much lower levels. Gomez and Hsia (1968) incubated slices of adult human male inguinal skin, neonatal foreskin and female abdominal skin with androstenedione and found that all skin samples produced similar products. These products included
testosterone, DHT, androstenedione and androsterone. 5β metabolites were not produced. Glickman and Rosenfield (1984) found that scalp and pubic hair follicles from normal women metabolized androstenedione to androstenedione, and to a lesser extent, testosterone. Perel et al (1986) reported that human breast adipose tissue also produced testosterone and DHT from androstenedione, but the major products were androstenedione and androsterone.

These data show that both testosterone and DHT are produced from the metabolism of androstenedione, and that these androgens are produced by liver as well as many peripheral tissues.

While various tissues may produce androgens from the metabolism of androstenedione in vitro, this pathway must also be shown in vivo before androstenedione could be considered as a major androgen precursor.

When androstenedione (100 mg) was taken orally by normal female volunteers, serum levels of testosterone became elevated within 90 minutes of administration (Mahesh and Greenblatt 1962). Similarly, intravenous injection of isotopic androstenedione (Bardin and Lipsett, 1967) showed that 49% of serum testosterone in normal women arises from androstenedione metabolism. These data suggest that in vivo metabolism of androstenedione can result in elevated androgen production. Since hirsutism is one manifestation of elevated androgen production in women, the occurrence of
hirsutism should be marked by elevated androstenedione levels if androstenedione is a major source of these androgens.

Bardin and Lipsett (1967) reported a mean plasma androstenedione level in normal women of 5.2 nmol/L. These levels were significantly lower than those measured in women presenting with idiopathic hirsutism (hirsutism occurring in the absence of polycystic ovarian syndrome: 9.8 nmol/L) or hirsute women with PCO (9.8 nmol/L). Since hirsutism is the result of elevated DHT stimulation within skin, these data suggest that androstenedione may be a precursor for DHT production. It is important to note however, that significant overlap in androstenedione levels exists between normal and hirsute groups. Meikle et al (1979) found that 15 out of 30 women with idiopathic hirsutism had androstenedione levels in the normal range. Toscano et al (1982) showed that 6 of 24 hyperandrogenic hirsute women had androstenedione levels in the normal range and Lobo et al (1983) showed that 6 of 12 hirsute PCO women had androstenedione levels in the normal range. These data suggest that androstenedione may be elevated in hirsute women as a group, but is not an accurate discriminator on an individual basis.

Both the ovary and adrenal produce androstenedione. To determine which of these tissues is the source of elevated androstenedione production in hirsute women, Kirschner et al
(1976) suppressed adrenal production of androstenedione in idiopathic hirsute (IH) women by administration of dexamethasone (2 to 4 mg/day) over a four to five day period. They found decreased androgen levels in only 50% of the IH group. Those women with the highest hirsutism scores were less likely to show suppression. However, Meikle et al (1979) showed that 3 weeks of dexamethasone treatment (0.75 mg/day) reduced androstenedione levels in most IH women to within the normal range. Similarly, Lachelin et al (1982) showed that androstenedione levels in women with PCO could be suppressed to normal levels (5.2 nmol/L) with dexamethasone (1.0 mg/day for 30 days) but that this inhibition was only temporary. Androstenedione levels gradually rose back up to pretreatment values following the first week of treatment. Cortisol levels did not rise over this same time interval which lead Lachelin et al to suggest that adrenal steroidogenesis was still inhibited and that ovarian production of androstenedione was increasing over this time period. Given that ACTH levels were not determined or that adrenal androgen production was not measured directly, it is difficult to assess the actual adrenal contribution to androgen production under this experimental regime. If adrenal androgen production is controlled by a factor other than ACTH (as suggested by Parker 1983), then recovery of androgen production in the
absence of such a recovery in cortisol levels may readily occur.

While increased ovarian production of androstenedione could explain the results obtained by Lachelin et al, others have failed to see such a recovery in androstenedione levels following dexamethasone treatment. Meikle et al (1986) reported that androstenedione levels remained suppressed for up to 3 months of dexamethasone (0.5 to 0.75 mg/day) treatment.

The **physiologic** relevance of androgen production from androgen precursors is dependent upon the compartment(s) where this metabolism occurs. The **in vitro** data presented at the beginning of this section show that peripheral tissues are capable of making androgens from androstenedione. This suggests that androgen production can be controlled at the individual tissue level. However, if significant hepatic production and secretion of androgens were to occur, the resulting increase in circulating androgen levels might over ride the differences seen in tissue production. As a result, hepatic production of androgens from precursors might result in generalized androgenization. The role of the liver in androgen metabolism is therefore an important one.

Rivarola et al (1976) determined the splanchnic extraction of androstenedione and the percent transfer of serum androstenedione to serum testosterone in men and women
during infusion of $^{14}$C-androstenedione under steady state conditions. They found that over 80% of the infused androstenedione was removed from the serum after only one passage through the splanchnic compartment, but that conversion of androstenedione to testosterone was only 16% of the total transferred. This suggests that while the splanchnic compartment efficiently removes androstenedione from the circulation, it does so by converting it to some metabolite other than testosterone.

Summary: Androstenedione can be metabolized to testosterone and dihydrotestosterone by many different tissues. The tissue which metabolizes the greatest percentage of androstenedione is the liver, and this metabolism results in the production of steroids other than testosterone. Therefore, androstenedione can act as a precursor of androgens, but this is not its major route of metabolism.

Due to the significant overlap in androstenedione levels between hirsute and non-hirsute women, this androgen precursor is a poor discriminator between these two groups.
ii. Dehydroepiandrosterone

DHEA, like androstenedione, is elevated in hyperandrogenic women. Lobo et al (1983) reported that hirsute PCO women had significantly higher DHEA levels than normal women. Abraham et al (1975) determined that the adrenal was the source of this increased DHEA production by showing that DHEA levels dropped following the administration of dexamethasone (2 mg/day for 5 days, controls not tested) while human chorionic gonadotropin (hCG: a stimulus for ovarian steroidogenesis) failed to elevate DHEA levels. This suggests that adrenal steroidogenesis contributes more to the circulating levels of DHEA in hirsute women than does ovarian steroidogenesis. This is supported by the findings of Lachelin et al (1982) who failed to see elevated DHEA levels in 4 hirsute women with polycystic ovaries.

While DHEA levels may be elevated in hirsute women as a group, Abraham et al (1975) showed that 50% of the women in their hirsute group had DHEA levels within the normal range. This suggests that DHEA is not a useful serum marker for the occurrence of hirsutism.

The fact that DHEA levels are elevated in hirsute women as a group does not necessarily mean that DHEA contributes to the elevated production of androgens in such women. If
DHEA is not metabolized to testosterone and/or DHT in vivo, there could be no direct cause and effect relationship between DHEA levels and the development of hirsutism. The DHEA levels would then simply reflect elevated adrenal steroidogenesis and the hirsutism would be the result of elevated adrenal androstenedione production.

To determine whether DHEA actually contributes directly to androgen production, Horton and Tait (1967) infused DHEA into normal male and normal female volunteers and then determined the transfer constants between DHEA and androstenedione as well as between DHEA and testosterone. The average value for the transfer constant between DHEA and androstenedione (i.e. the % of DHEA converted to androstenedione) was 6 % with no difference seen between males and females. The average value for the transfer constant between DHEA and testosterone was only 0.7%.

These data suggest that DHEA is only a minor source of precursor for serum androstenedione and testosterone. However, when Mahesh and Greenblatt (1962) gave an oral dose of DHEA to two subjects, they found that the levels of serum testosterone increased by a factor of 3 to 4, suggesting that DHEA could be a significant precursor of testosterone. The dose of DHEA used in the Mahesh and Greenblatt study was quite large however (100 mg) and the resulting serum levels of DHEA were not determined. High serum levels of DHEA
would result in a significant elevation of testosterone even with a small transfer constant.

While DHEA metabolism in vivo may not result in significant production of testosterone or DHT, Lindberg et al (1966) measured the appearance of radioactive glucuronide and sulfate metabolites in human serum following injection of either $^3$H- or $^{14}$C- DHEA. Serum levels of glucuronide metabolites peaked within 30 minutes following DHEA injection and were cleared from the circulation by 4 hours post administration. Sulfate levels rose more slowly, peaking after 60 to 90 minutes and remained high over the 4 hour test period. The major sulfate formed from DHEA was DHEAS with androsterone sulfate and etiocholanolone sulfate as minor products. When minces of human benign prostate tissue were incubated with DHEA however, no DHEAS was produced (Harper et al 1974). The major metabolites formed were androstenediol, $5\alpha$-androstane-3\beta,17\beta-diol, DHT, $5\alpha$-androstane-3\alpha,17\beta-diol and androsterone.

In summary, these data show that metabolism of DHEA does not contribute significantly to the circulating levels of testosterone or DHT. Androgen conjugates are produced from the metabolism of DHEA, with DHEAS being the major such conjugate.

Due to the significant overlap in DHEA levels between hirsute and non-hirsute women, this androgen precursor is not a useful marker of peripheral DHT metabolism.
iii. Dehydroepiandrosterone Sulfate

The data presented above suggest that the development of hirsutism is accompanied by elevated adrenal steroidogenesis. Since dehydroepiandrosterone sulfate (DHEAS) is the major product of adrenal steroidogenesis, it would seem likely that DHEAS levels would also be elevated in hirsute women.

Lobo et al (1983) measured DHEAS levels in normal women, non-hirsute women with PCO and hirsute women with PCO. They found that PCO women as a group had DHEAS levels which were higher than normals. In addition, they found that the levels of DHEAS in hirsute PCO women were significantly greater than were the levels in non-hirsute PCO women suggesting a role for DHEAS in the development of hirsutism. However, as was noted above for androstenedione and DHEA, DHEAS levels show significant overlap between the hirsute and non-hirsute groups. Abraham et al (1975) and Lobo et al (1983) both found that 50% of their hirsute women had DHEAS levels in the normal range while Toscano et al (1982) found that 17 of 24 hirsute women had DHEAS levels similar to those of normal women.

As was discussed with DHEA (above), increased DHEAS levels in hirsute women need not signify a cause and effect relationship. For such a relationship to be established, it
must be shown that DHEAS can be metabolized to testosterone or DHT.

Harper et al (1974) infused tritiated DHEAS into three men with benign prostatic hyperplasia 30 minutes prior to prostatectomy. The prostate tissue was then analyzed for metabolites of the DHEAS and they found that most of the activity remained as DHEAS but measurable amounts of tritiated DHEA, testosterone, DHT and androsterone were found as well. In addition, androsterone sulfate and etiocholanolone sulfate were produced.

These data suggest that DHEAS is not readily metabolized to testosterone or DHT. However, DHEAS may still contribute to the circulating levels of these androgens, since serum DHEAS levels are very high and even a small percent conversion could result in significant androgen production.

Due to a significant overlap in DHEAS levels between hirsute and non-hirsute women, this androgen precursor is not an appropriate marker of peripheral DHT metabolism.
B/ Potent Androgens

The potent androgens include testosterone and DHT. Both of these steroids bind with high affinity to the androgen receptor. As such, both exert androgenic activity, but the degree of activity which each exerts varies between tissues.

i. Testosterone

In the skin and prostate, testosterone’s androgenic actions are expressed only after it is converted to DHT. In other tissues, such as brain and muscle, testosterone itself appears to be the major androgen. For the purposes of this thesis, testosterone will be considered as a precursor of DHT formation and as such, will be reviewed in a manner similar to that of the previously described androgen precursors.

Bardin and Lipsett (1967) reported an average plasma testosterone level in normal women of 1.39 nmol/L. This value was significantly lower than that found in IH (2.8 nmol/L) and hirsute PCO (3.61 nmol/L) women. Normal women also had a lower blood production rate (0.8 μmol/day) and lower metabolic clearance rate (590 L/day) than the IH (2.9 μmol/day; 1080 L/day) and PCO (4.1 μmol/L; 1110 L/day)
women. These data suggest that production of testosterone is elevated in hirsute women as a group. Not all hirsute women have elevated testosterone levels however. Toscano et al (1982) reported that 14 of 24 hirsute women had testosterone levels in the normal range. Lobo et al (1983) determined that 8 of 13 hirsute women had normal levels of testosterone while Abraham et al (1975) found that 60% of hirsute women had levels of testosterone within the range of the normal group.

The DHT responsible for the development of hirsutism in these women could arise directly from the 5α-reduction of testosterone. Not all testosterone is available for 5α-reduction however. Most testosterone is bound to the carrier protein sex hormone binding globulin (SHBG). Only the unbound (or 'free') testosterone is available for metabolism. Horton et al (1982) measured total testosterone and free testosterone in hirsute and normal women. They found that free testosterone was a better discriminator between these two groups than was total testosterone. There was still a significant overlap in free testosterone levels between groups however, with 25 % of hirsute women having free testosterone levels in the normal range.

While the data presented above suggest that elevated testosterone levels contribute to the development of hirsutism, other data fail to support this. Lobo et al (1983) reported that testosterone levels in PCO women did
not accurately discriminate between hirsute and non-hirsute PCO women. They concluded that hirsutism is characterized by elevated testosterone levels, but that excess testosterone did not necessarily cause this hirsutism. This conclusion may have been inaccurate however. While the mean and standard deviations of the various groups are not given, scattergrams of the data are presented. The average testosterone levels in the hirsute PCO group was higher than the non-hirsute PCO group, but the hirsute group also had a much greater variance. This made comparisons between groups difficult. Perhaps a larger sample size (they had 12 in each group) would show a significant difference between the hirsute and non-hirsute groups.

Since testosterone is produced from direct ovarian and adrenal secretion as well as from peripheral androstenedione metabolism, any one of these tissues could be responsible for elevated testosterone production in hyperandrogenic women. Lachelin et al (1982) reported diurnal oscillations in testosterone levels synchronous with cortisol secretion. They also reported that testosterone levels in PCO were inhibited by dexamethasone but, like androstenedione (see androstenedione discussion), this suppression was gradually lost. Steinberger et al (1984) also reported that dexamethasone inhibited testosterone levels in hyperandrogenic women (women with acne or hirsutism or both) and that this suppression was correlated to the degree of
DHEAS suppression (i.e. adrenal suppression). Unlike these other reports, Kirschner et al (1976) found that dexamethasone suppressed testosterone levels in only one half of the hirsute women tested, and the degree of suppression in those women who responded to treatment was only minimal. While Kirschner's findings suggests that the ovary is the principal source of testosterone in hirsute women, the conditions of the suppression test may have been sub-optimal. The dose of dexamethasone was high enough (2-4 mg/day) and the period of treatment should have been long enough (4 to 5 days) for measurement of some effect. However, adrenal function was not tested in these women to determine whether this treatment was effective in blocking adrenal steroidogenesis. DHEAS and/or cortisol levels should have been measured with the degree of testosterone suppression adjusted within individuals against these levels to correct for individual responses to this treatment.

The data presented above suggest that testosterone can contribute to the development of hirsutism. This likely results from the conversion of testosterone to DHT within the skin. To determine the degree of peripheral testosterone metabolism, Ishimaru et al (1978a) measured the splanchnic and extrasplanchnic clearance of tritiated testosterone in men. The splanchnic clearance was 638 L/day while the extrasplanchnic clearance was 314 L/day. In addition, Rivarola et al (1967) showed that 44% of infused
tritiated testosterone was removed from the serum by the liver during the first passage. These data suggest that while most of the circulating testosterone is rapidly metabolized by the liver, approximately one third is metabolized by the extrasplanchnic compartment, including skin.

To determine whether the production of DHT from testosterone is elevated in hirsute women, the degree to which testosterone is metabolized to products other than DHT by competing metabolic pathways must be assessed. Rivarola et al (1967) determined that less than 0.02 % of infused $^3$H-testosterone was converted to androstenedione. Mahoudeau et al (1971) reported that 4 % of $^3$H-testosterone was converted to DHT in both normal men and hirsute women. Moghissi et al (1984) showed that the conversion ratio of testosterone to androstanediol glucuronide (Adiol-G) was less than 5 % while the conversion ratio of testosterone to DHT-G was 70 %, making DHT-G the major metabolite of testosterone. This conclusion is in direct contradiction to the conclusions which will be drawn from the experiments presented in this thesis. These discrepancies are dealt with in the discussion section, but a brief comment about this issue is necessary at this point. It is clear that one cannot separate DHT-G from Andros-G using the chromatography system employed in the Moghissi paper. The separation techniques used in this thesis readily separate these two conjugates and based upon
the data presented in Experiment 1 of this paper, DHT is readily metabolized to Andros-G but not DHT-G. For this reason, it is suggested that Moghissi et al actually measured Andros-G, but misidentified it as DHT-G. This conclusion is supported by the work of Horton et al (1963) who found that 40% of an injected dose of tritiated testosterone appeared in the glucuronide and sulfate conjugates of androsterone and etiocholanolone. Similarly, Zumoff et al (1971) reported that 48% of $^{14}$C-testosterone was recovered from the etiocholanolone and androsterone fractions in urine (i.e. steroid conjugates). Deslypere et al (1981) also found that the major urinary metabolites of testosterone were Andros-G and etiocholanolone-G, accounting for over 65% of injected $^{14}$C-testosterone. An additional 10% of the administered steroid was accounted for in the androsterone-sulfate, etiocholanolone-sulfate and Adiol-G fractions. Lindberg et al (1966) reported that $^{14}$C-testosterone was metabolized to glucuronides and sulfates rapidly, appearing in the urine within 30 minutes of injection for the glucuronides and 60 to 90 minutes for the sulfates.

These data show that testosterone is efficiently metabolized to androgen conjugates. Whether the production of androgen conjugates from the metabolism of testosterone requires the production of DHT as an intermediate step is not addressed in any of this work.
The information given above suggests that elevated testosterone levels accompany the development of hirsutism, that most of the circulating testosterone is rapidly metabolized by the splanchnic compartment and that androgen conjugates are the principal products of testosterone metabolism. Since the splanchnic compartment metabolizes the greatest percentage of circulating testosterone, the metabolites identified above most likely reflect the metabolic pathways present in the splanchnic compartment. It is not clear what the products of peripheral testosterone metabolism are however. Perhaps the peripheral tissues bypass the production of DHT altogether, preferring to enter the 17-ketosteroid metabolic route (Fig 1, pg 11). For this reason, it is important to determine in vitro what the metabolites of testosterone metabolism by peripheral tissues are. Bruchovsky and Wilson (1968) infused $^3$H-testosterone intravenously into rats and found that within 1 minute of administration, 90% of prostatic testosterone had been converted to metabolites. The major product was DHT, with Adiol, androsterone and androstenedione also produced. Baulieu et al (1968) also found that the major product of testosterone metabolism by ventral prostate was DHT with some androsterone and Adiol also being produced. Pike et al (1970) infused testosterone into men undergoing prostatectomy due to benign prostatic hyperplasia. Upon removal of the prostate (20 min following infusion), they
found that the major testosterone metabolites were DHT and androstenedione. In a similar experiment, Harper et al (1974) also showed that DHT was the major product, with androsterone and Adiol also produced.

Dijkstra et al (1987) examined testosterone metabolism by different skin layers from human balding scalp. They found that epidermal tissue and hair follicles metabolized testosterone to androstenedione, while deeper regions containing sebaceous glands produced mainly androsterone. Cultured hair follicle keratinocytes produced androstenedione and androsterone in similar amounts. However, when total skin was examined, the major product of testosterone metabolism was found to be androsterone.

Lobo et al (1987) showed that DHT-G and Adiol-G are two major testosterone metabolites produced by genital skin, with skin from males producing more conjugate than skin from females. Again, a question arises as to the actual identity of the peak labeled as DHT-G.

Jenkins and Ash (1973) measured testosterone metabolism in suprapubic skin of normal, IH and PCO women. They found that skin from normal women metabolized testosterone to DHT, androstenedione, androsterone and androstanedione. Male skin showed the same overall pattern of metabolism. Skin from hirsute women produced more DHT and androstenedione than did the skin from normals. Gomez and Hsia (1968) also reported that the major products of testosterone metabolism
by human skin were androstenedione, DHT, androsterone and androstanedione. In addition, they found significant production of epiandrosterone.

Milewich et al (1985) incubated slices of human lung with $^3$H-testosterone and found that, like skin, the major products were androstenedione, androstanedione, epiandrosterone and androsterone.

Chung and Coffey (1977) looked at the production of androgen glucuronides from testosterone by various rat tissues. They found that ventral prostate, seminal vesicles, coagulating gland, liver and kidney were all capable of forming androgen glucuronides. Testosterone glucuronide was the major product of the liver and kidney, while the accessory sex organs produced primarily androstanediol 17-glucuronide (one of the possible isomers of Adiol-G).

In summary, testosterone levels are elevated in hirsute women. This testosterone is produced from the metabolism of both adrenal and ovarian steroids, and the amount derived from either organ varies within individuals. The splanchnic compartment rapidly metabolizes the greatest percentage of the circulating testosterone, but peripheral tissues metabolize up to one third of that available. Different tissues produce different products from such metabolism, but prostate and skin readily make DHT. DHT production by skin does not appear to occur in the hair follicle.
Due to significant overlap in testosterone levels between the hirsute and non-hirsute groups, serum testosterone levels are not a useful marker of peripheral DHT metabolism.

This thesis deals with the relationship between androgen glucuronide formation and peripheral DHT metabolism. The previous sections have dealt with the pathways of metabolism leading to DHT production. The remaining sections will deal with the metabolic pathways leading to the elimination of DHT, including those pathways leading to the production of androgen-glucuronides.

ii. Dihydrotestosterone

This thesis examines the relationship between androgen glucuronide formation and peripheral DHT metabolism. This relationship is examined since androgen conjugates are believed to more accurately reflect DHT metabolism by peripheral tissues than do circulating levels of DHT itself.

The poor correlation between serum DHT levels and degree of peripheral androgen metabolism has been shown by several different groups. Toscano et al (1981) measured plasma levels of DHT in normal and hirsute women. Unlike many of the other steroids mentioned above, the levels of
DHT were not significantly different between these two groups (0.59 ± 0.2 nmol/L in normal and 0.79 ± 0.4 nmol/L in hirsute women) suggesting that DHT was only a poor indicator of hirsutism. Similarly, Lobo et al (1983) found that serum levels of DHT did not differ between normal women, non-hirsute PCO women and PCO women with hirsutism. However, Kuttenn et al (1977) reported that hirsute women had a mean plasma DHT level of 1.21 nmol/L which was significantly higher than that measured in normal women (0.62 nmol/L).

These findings suggest that a difference in serum DHT levels may exist between hirsute and non-hirsute women, but that this difference is only minor and may only be seen when subject selection favours the two extremes. This suggestion has scientific merit since recent work (Lookingbill et al 1990) has shown that androgen levels show racial differences. They found that androgen levels in caucasian women exceed those of oriental women. Based on this finding, it becomes necessary to ensure that both test and control groups have equal racial representation or else the results may be skewed. It is difficult to determine whether the differences seen between Kuttenn’s findings and those of Lobo or Toscano are related to subject selection since the pertinent background information about the subjects is not given.
The data presented above suggest that either DHT production is normal in hyperandrogenic women, or that production is elevated and that this increase is balanced by increased clearance. Mahoudeau et al (1971) reported the metabolic clearance rate (MCR) for DHT in normal and hirsute women. The MCR was 243 L/day for normal women. Hirsute women had an MCR of 517 L/day which suggests that the lack of difference in serum DHT levels between normal and hirsute women results from an increased DHT clearance. Since this is the case, the blood production rate of DHT must be greater in hirsute than normal women since serum levels are similar between these groups. Ito and Horton (1971) reported a blood production rate for DHT of 0.2 µmol/day in normal females. Samojlik et al (1984) reported obese, non-hirsute, normally menstruating women had a DHT blood production rate of 0.6 µmol/day which was higher than normal women, while the rate of production in IH women was even greater, being 1.3 µmol/day. From these data it can be concluded that hirsute women produce more DHT than do normal women, but that hirsute women clear DHT more rapidly than normal women. This suggests that circulating levels of DHT are more rigorously controlled than are the levels of the steroids previously reviewed. As a result, serum DHT levels are not an adequate marker of peripheral DHT metabolism.

Since serum DHT levels do not adequately reflect the
metabolism of DHT by peripheral tissues in either normal or hirsute women, this information must be derived directly.

Glickman et al (1984) examined DHT metabolism by hair follicles. They found that scalp hair from normal women metabolized DHT to androstanedione at a rate 40 times greater than was seen for conversion to Adiol. Pubic hair from normal women and normal men also metabolized DHT to androstanedione.

To determine how significant such extrasplanchnic metabolism of DHT is to the overall metabolism of DHT, Ishimaru et al (1978a) infused $^3$H-DHT into normal men and measured the percent extraction and serum clearance by the splanchnic and extrasplanchnic compartments. It should be noted that bile was not collected, therefore hepatic clearance via the bile cannot be determined. Given this limitation, they found that the splanchnic compartment extracted 38% of the infused DHT. They also showed that the splanchnic compartment cleared 343 L/day while the extrasplanchnic compartment cleared 421 L/day, suggesting that the majority of circulating DHT is in fact metabolized by peripheral tissues.

The products from such clearance include androgen conjugates. Moghissi et al (1984) measured the production of androgen glucuronides in serum following infusion of DHT. The major products were Adiol-G (39%) and DHT-G (or possibly Andros-G: 30%). Mauvais-Jarvis et al (1968)
measured the urinary products of DHT metabolism and found that the major product was Andros-G. Other products included Adiol-G, androsterone sulfate and isoandrosterone sulfate.

From the above data it has been shown that serum DHT levels do not correlate well with the development of hirsutism. As such, serum DHT levels do not adequately reflect the degree of androgenicity within skin. This may result from the conversion of DHT to other steroids prior to release from the tissues.

The poor correlation between serum DHT levels and the development of hirsutism indicate that serum levels of this androgen can not be used as a marker of peripheral DHT metabolism.

C/ Unconjugated Androgen Metabolites

Androstanediol binds to the androgen receptor but does so with much lower affinity than either testosterone or DHT. Androsterone has a ketone group at the 17-carbon position and as a result, can not bind to the androgen receptor. These two steroids will therefore be referred to as androgen metabolites rather than potent androgens since their production from either testosterone or DHT will decrease (in the case of Adiol) or terminate (in the case of androsterone) the androgen signal.
i. Androstanediol

The term 'Adiol' usually refers to the compound $5\alpha$-androstan-3α,17β-diol. However, other isomeric forms of this compound exist, including $5\alpha$-androstan-3β,17β-diol. Toscano et al (1981) reported that the 3β isomer is the major form of serum Adiol in both men and women, being present in levels twice that of the 3α isomer. In addition, the levels in men (3α 0.99 nmol/L; 3β 1.88 nmol/L) were two times those of women (3α 0.38 nmol/L; 3β 0.82 nmol/L). While castration reduced the levels of both isomers, the reduction of the 3β isomer was much greater than was that of the 3α. Habrioux et al (1978) also showed that the 3β isomer is the major form of serum Adiol not only in normal men and normal women but in hirsute women as well. Their values were different from Toscano's however, having a 3β to 3α ratio closer to 4. These data suggest that the preferred pathway of DHT metabolism is via the 3β-ketosteroid reductase enzyme rather than the 3α-ketosteroid reductase enzyme.

As was mentioned above, the abbreviated term Adiol will represent the 3α-isomer, since this is the isomer measured most often. When a different isomer is being discussed, the chemical description of that isomer will be given.

Since Adiol is a direct product of DHT metabolism, the elevated DHT production measured in hyperandrogenic women
(see above) should result in elevated serum Adiol levels.

Horton et al (1982) failed to show a significant elevation in Adiol levels between normal women and women with IH. However, the hirsute women used in their study were all classified as mildly IH. The difference between normal women and women with severe IH or PCO was examined by Toscano et al (1982). They reported that 3α but not 3β Adiol was elevated in hirsute women. Similarly, Samojlik et al (1984) determined that Adiol was significantly higher in hirsute women when compared to normal or obese groups. In addition, Lobo et al (1983) showed that Adiol was higher in hirsute PCO women than in non-hirsute PCO women and that both PCO groups had levels which were greater than normal women.

In the Toscano report, only one of 23 hirsute subjects had Adiol levels within the normal range. However, Lobo et al (1983) showed that 5 of 11 hirsute PCO women had normal levels of Adiol. Similarly, Meikle et al (1979) showed that approximately 50% of IH women tested had levels of Adiol which were within the normal range.

To determine whether Adiol is an end product of androgen metabolism, or whether it itself undergoes further metabolism, Verhoeven et al (1977) examined the metabolism of Adiol by various rat tissues. They found that kidney converted Adiol to DHT (25 % of products), androsterone (20 %) and androstanedione (15 %). An additional 9 % of the
products were present as androgen conjugates. DHT was the principal Adiol metabolite (69 %) produced by prostate tissue with all other products present in low amounts (3-5 % each). Liver produced primarily polar and conjugated androgens (86 % combined). Milewich et al (1985) found that the major product of Adiol metabolism by human lung was DHT. Other products included androsterone, isoandrosterone, androstanedione and four polar metabolites. These data show that Adiol is not an end product of androgen metabolism and as such would not be an ideal marker for peripheral DHT metabolism since its levels could be controlled by factors other than peripheral DHT production. In addition, it is important to note that the major product of Adiol metabolism within many different tissues was DHT. This is an important point since conversion of Adiol to DHT would result in an amplification of the androgenic signal within such tissues. When DHT is metabolized to Adiol, the strength of the androgen signal would decrease. Back conversion of this Adiol to DHT would return the androgen signal back to its original strength. In this way a shuttle would be formed that would alternately diminish then increase repetitively the level of androgen stimulation within these tissues.

Due to significant overlap in Adiol levels between the hirsute and non-hirsute groups, serum Adiol levels are not a useful marker of peripheral DHT metabolism.
ii. ANDROSTERONE

Androsterone is produced from DHT by the combined enzymatic actions of 17-hydroxysteroid dehydrogenase and 3-ketosteroid reductase (Fig 1, pg 11). Both enzymes are present in various tissues (as evidenced by the metabolic pathways already noted above), suggesting that androsterone could be a major product of DHT metabolism. Since androsterone has a ketone group at the 17-carbon position, it does not bind to the androgen receptor and as such, has no intrinsic androgenic activity. Therefore, conversion of DHT to androsterone would represent an important step in androgen signal attenuation. For this reason, data describing the production of androsterone in normal men and women as well as hyperandrogenic women will be reviewed.

Hellman et al (1977) measured the level of plasma androsterone in men over a 24 hour period and found the average value to be 1.89 nmol/L. The half life for androsterone was calculated to be 25 minutes and the metabolic clearance rate was 4050 L/day. Meikle et al (1979) reported that the levels of androsterone were similar between men (1.1 nmol/L) and women (1.17 nmol/L). Belanger et al (1986a) found that while plasma levels of testosterone, DHEA, DHT, and Adiol declined with age in adult men, androsterone levels actually increased in these
groups (from 1.41 nmol/L at 20-35 years of age, to 2.89 nmol/L at 55-70 years of age).

Androsterone itself can undergo further metabolism. Milewich et al (1985) found that human lung tissue metabolized androsterone primarily to androstanedione while Adiol and DHT were produced in smaller amounts. Four polar compounds were also identified. The major end products of androsterone metabolism however, are the glucuronide and sulfate conjugates of androsterone. Rosenfeld et al (1980) found that 74-100 % of orally administered androsterone is rapidly metabolized to these conjugates.

While androsterone conjugates may be the major end products of androsterone metabolism, the conversion of androsterone to androstanedione also occurs readily. Fukushima et al (1967) have shown that the 3-hydroxyl of androsterone undergoes extensive dehydrogenation and resynthesis (i.e. the carbon skeleton shuttles between androstanedione and androsterone). This is similar to what is seen with Adiol and DHT (both equilibria are catalyzed by the same enzyme, 3-ketosteroid reductase). Since a major product of DHT metabolism within many peripheral tissues (see DHT section above) is androstanedione, androsterone must then also be a major DHT metabolite. Hyperandrogenism in women is accompanied by elevated androsterone levels. This elevated production of androsterone appears to result from elevated adrenal steroidogenesis. Brochu et al (1987)
showed that androsterone levels were elevated in those hirsute women having high DHEAS levels, while androsterone levels were normal in hirsute women which had normal DHEAS. However, there is evidence which suggests that the gonad contributes to androsterone production as well, since Belanger et al (1986b) found that androsterone levels were lower in castrated men than in men which were not castrated.

In summary, androsterone may be an important physiological metabolite of DHT. However, Meikle et al (1979) showed that 22 of 30 hirsute women had androsterone levels within the normal range, suggesting that this androgen metabolite is a poor marker of peripheral DHT metabolism.

D/ ANDROGEN GLUCURONIDES

Androgen conjugates are much more water soluble than are the unconjugated androgens. The androgen conjugates are therefore more readily excreted in the urine than are the unconjugated androgens. For this reason, the conjugation of androgens is considered to be a final step in the elimination of the androgens from the circulation. Since this thesis addresses the relationship between peripheral DHT metabolism and the production of androgen glucuronides,
the literature pertaining to androgen glucuronide production will be reviewed.

i. Testosterone Glucuronide

DHT is produced from testosterone by the irreversible enzymatic step of 5α-reduction. If testosterone is glucuronidated prior to 5α-reduction, then production of unconjugated DHT from testosterone would decrease. This would be an important mechanism by which DHT levels could be controlled. For this reason, the production of testosterone glucuronide will be reviewed.

Tresguerres et al (1977) reported that the serum levels of testosterone glucuronide are highest in men (4.5 nmol/L), intermediate in hirsute women (2.93 nmol/L) and lowest in normal women (1.01 nmol/L). Belanger et al (1986a) reported that plasma testosterone glucuronide levels in men increased with age, rising from a value of 2.2 nmol/L in men aged 20-35 years, to a value of 4.7 nmol/L in men aged 55-70 years. However, Wright et al (1985) reported that urinary production of testosterone-G decreased with age. It should be noted that the levels which Belanger presented for his young group appear to be much lower than are the levels presented by Tresguerres, who also studied young men (20-28 years of age). If Belanger's sampling population was skewed, or they failed to properly correct for procedural
losses in their assay (as is the case for their Adiol-G assay), then they would have had an artificially low reading for this young group. The rise in levels seen with age may only be artifactual under such conditions, since the levels they report for their older group match closely the values reported by Tresguerres for young men.

Tresguerres et al (1977) found that testosterone glucuronide levels in hypogonadal men (ages 52-65) were lower (2.4 nmol/L) than normals (4.5 nmol/L). Similarly, Belanger et al (1986b) reported that testosterone glucuronide levels dropped from 5.38 nmol/L in intact men to 1.5 nmol/L in castrate men. These data suggest that testosterone glucuronide levels in men result primarily from the metabolism of steroids produced by the testis. However, when Tresguerres et al (1977) infused 1000 IU of hCG, there was no effect on serum testosterone glucuronide levels. These measurements were made during the 3 hours which followed the infusion however, and this may not have been enough time to generate a significant effect.

Testosterone glucuronide does not appear to be an important metabolite of testosterone in vivo, since several reports have shown that less than 1% of administered testosterone was metabolized to testosterone glucuronide (Baulieu and Mauvais-Jarvis 1964; Robel et al 1966; Horton et al 1965). However, testosterone glucuronide does appear to be a significant product of testosterone metabolism in
vitro. Dog liver converted 15-16 % of testosterone to testosterone glucuronide while rat liver converted 7-8 % (Fishman and Sie, 1956). Chung and Coffey (1977) reported that testosterone-G was produced efficiently from testosterone by rat kidney (40 %), liver (39 %) and seminal vesicle (29 %). In addition, Chung and Coffey (1978) found that testosterone glucuronide was the major testosterone metabolite produced by normal human prostate tissue. Korenman et al (1964) infused isotopically labeled androstenedione into humans and found that testosterone glucuronide is also a product of androstenedione metabolism.

In summary, testosterone glucuronide is present in serum of men and women. Elevated levels of testosterone glucuronide coincide with the occurrence of hirsutism. Since testosterone glucuronide formation decreases the amount of testosterone converted to DHT, this may be an important mechanism in the control of DHT levels and hence, androgen expression. It would be interesting to see whether the severity of hirsutism would increase if testosterone glucuronide formation in hirsute women was artificially blocked.
ii. Dihydrotestosterone Glucuronide

Glucuronidation of DHT would result in a direct, rapid and virtually irreversible termination of the androgenic actions of this steroid. As such, this metabolic pathway may contribute significantly to the elimination of DHT by peripheral tissues. The production of DHT-G will therefore be reviewed.

Belanger et al (1986b) were unable to detect serum DHT-G levels in either intact or castrated men. However, Brochu et al (1987) did detect DHT-G in the serum of normal and hirsute women. Normal women had an average level of 0.8 nmol/L, IH women (defined by Brochu as women who are hirsute but have normal DHEAS levels) had an average level similar to normals while hyperandrogenic hirsute women (defined by Brochu as women who are hirsute and have high DHEAS levels) had an average DHT-G value of 1.4 nmol/L. This was significantly higher than were the values measured in the normal and IH groups. These data suggest that the increased DHT-G levels reflected the metabolism of adrenal steroids.

Moghissi et al (1984) found that infused testosterone was metabolized much more readily to DHT-G than to either testosterone glucuronide or Adiol-G. Ishimaru et al (1978b) showed that conversion of testosterone to DHT-G occurs in the splanchnic compartment, and that this conjugation of DHT
occurs before it mixes with the peripheral DHT pool. These data suggest that the splanchnic compartment eliminates a significant fraction of testosterone before it can contribute to the production of DHT by peripheral tissues. As was mentioned in the section on testosterone metabolism (pg 40-41), it is not clear whether the product they measured was in fact DHT-G or Andros-G, but for the purposes of this particular discussion, this point is not important. What is important is that there is a splanchnic mechanism for the metabolism of testosterone which does not contribute DHT to the circulation.

While the splanchnic compartment may be capable of producing DHT-G from the metabolism of testosterone, it is the production of DHT-G by extrasplanchnic tissues which determines the significance of this metabolic pathway in terminating the DHT signal within peripheral tissues.

Chung and Coffey (1978) reported that the major metabolite of testosterone in hyperplastic human prostate tissue was DHT-G, with testosterone-G produced at only one sixth that amount. Similarly, Lobo et al (1987) showed that human genital skin produced DHT-G from testosterone, with skin from normal men producing 10 times the amount that skin from normal women produced. The rate of DHT-G production was greater than was the rate of Adiol-G production. Djoseland (1976) reported that DHT-G was one of the major testosterone metabolites produced by rat epididymis.
These data suggest that many different tissues are capable of metabolizing testosterone to DHT-G and as such, may be an important metabolic pathway for the elimination of DHT within peripheral tissues.

iii. Androstanediol Glucuronide

Many reports have shown that serum Adiol-G levels are elevated in hirsute women. In several studies, Adiol-G levels appear to correlate better with the degree of hirsutism than do serum DHT levels. For this reason, it has been suggested that peripheral tissues such as skin, metabolize DHT to Adiol-G. The greater the degree of hair follicle stimulation by DHT, the greater the production of Adiol-G within the skin. Therefore, the greater the degree of hirsutism, the higher the serum levels of Adiol-G. If the splanchnic compartment does not metabolize DHT to Adiol-G, then Adiol-G would be the ideal marker of peripheral DHT metabolism. The work presented in this thesis address the validity of this assumption. For this reason, the data describing Adiol-G production will be reviewed.

Androstanediol has hydroxyl groups at both the 3 and 17 carbon positions. Glucuronidation of Adiol can occur at either of these hydroxyls, producing either androstanediol 3-glucuronide (Adiol 3-G) or androstanediol 17-glucuronide (Adiol 17-G). However, Adiol-G values reported in the
literature usually refer to total Adiol-G. To obtain this value, the glucuronide is first removed from the Adiol-G molecule by enzymatic hydrolysis and then the amount of liberated Adiol is measured in a radioimmunoassay (RIA) specific for unconjugated Adiol. Unless otherwise stated, the term Adiol-G will refer to this total Adiol-G.

If serum Adiol-G levels represent peripheral DHT metabolism then the levels of Adiol-G should be elevated in hirsute women. Horton et al (1982) showed that Adiol-G levels in IH women (12.9 nmol/L) were not only higher than in normal women (0.9 nmol/L), but that only one woman in the IH group had a level which overlapped the normal range. This suggests that Adiol-G is a good discriminator between normal and IH women. Similarly, Kirschner et al (1987) showed that almost all IH women had serum Adiol-G levels which were higher (10.4 nmol/L) than normal women (2.5 nmol/L). Lobo et al (1983) reported the levels of Adiol-G in hirsute and non-hirsute PCO women. They found that the non-hirsute PCO group had an average serum Adiol-G level of 1.5 nmol/L which was not different from the level measured in the normal group (0.9 nmol/L). The hirsute PCO group, however, had a serum Adiol-G value of 11.7 nmol/L which was significantly higher than the normal and non-hirsute PCO groups.

These data suggest that Adiol-G is a good discriminator between hirsute and non-hirsute women. As such, Adiol-G
would be an ideal marker of peripheral DHT metabolism. However, there are other reports which dispute this high degree of discrimination between hirsute and non-hirsute groups based on Adiol-G levels (Brochu et al 1987; Paulson et al 1986; Habrioux et al 1978; Rittmaster and Givner 1988). This issue will be examined in much greater detail in the discussion section since resolution of this dispute is a central issue of this thesis.

Since this thesis addresses the issue of androgen-glucuronides as markers of peripheral DHT metabolism, it is important to know which tissues produce Adiol-G. Morimoto et al (1981) measured the specific activities of Adiol-G across the splanchnic compartment following infusion of isotopic DHT. No gradient for Adiol-G was detected across this compartment, suggesting that the liver does not contribute to Adiol-G production in man. Because of this finding, Adiol-G has generally been considered as a product of peripheral androgen metabolism only. Data presented in this thesis call this conclusion into question. The discussion section of this paper examines this issue and offers a model of Adiol-G production which is consistent with Morimoto's data as well as our own.

The data presented above suggest that Adiol-G is a marker of DHT metabolism by skin. To confirm this, in vivo production of Adiol-G from DHT must be shown. Mauvais-Jarvis et al (1968) injected normal men and women
with tritiated DHT and testosterone. They showed that tritiated Adiol-G was present in the urine of those subjects infused with DHT, but was not present in the urine of those subjects infused with testosterone. Similarly, Moghissi et al (1984) showed that the conversion of DHT to Adiol-G was very high (40%), while the conversion of testosterone to Adiol-G was much lower (less than 5% of the administered activity). These data show that Adiol-G is a marker of androgen metabolism, specifically DHT metabolism.

Should Adiol-G undergo extensive metabolism itself, then serum levels of Adiol-G would reflect not only its production from DHT but also the extent to which it is eliminated. Therefore, the degree to which Adiol-G is itself metabolized is an important factor in determining its usefulness as a marker of DHT metabolism. Greep et al (1986) determined that the conversion of Adiol-G (administered as isotopic Adiol 17-G) to unconjugated DHT was less than 1%. The conversion to DHT-G was a little higher but was still only 6%, suggesting that Adiol-G is not only a major product of DHT metabolism but may be an end product of such metabolism.

In summary, Adiol-G is believed to be produced from the metabolism of DHT by peripheral tissues. However, there is conflicting evidence over whether serum Adiol-G levels are an adequate marker of peripheral DHT metabolism.
iv. Androsterone Glucuronide

Andros-G is an androgen conjugate present in levels much higher than the corresponding Adiol-G values. For this reason it is hypothesized that Andros-G, like Adiol-G, is an important marker of androgen metabolism. The factors controlling the production of Andros-G will therefore be reviewed.

Belanger et al (1986a) measured Andros-G in plasma of young (20-35 years of age) and elderly (55-70 years of age) men and reported values of 49 nmol/L in the young group and 30 nmol/L in the elderly group. These values were much higher than were the corresponding Adiol-G values (17 nmol/L in the young men and 13 nmol/L in the elderly men). The level of Andros-G in normal women has been reported by Brochu et al (1987) to be 15 nmol/L which again is much higher than that of the corresponding Adiol-G level (4.3 nmol/L).

Like Adiol-G, elevated Andros-G levels are associated with hirsutism. Hirsute women with normal DHEAS levels had an average Andros-G value of 25.7 nmol/L while women with hirsutism associated with high DHEAS levels had levels of Andros-G which were even higher (72.9 nmol/L). Both groups had levels which were significantly greater than control (Brochu et al 1987).
Andros-G and its 5β isomer, etiocholanolone glucuronide (etiocholanolone-G), are the major androgen conjugates formed in vivo from the metabolism of testosterone (Baulieu and Mauvais-Jarvis 1964; Robel et al 1966; Deslypere et al 1981). This metabolic pathway appears to be blocked however if testosterone undergoes glucuronidation (Robel et al 1966), suggesting that the conversion of testosterone to Andros-G or etiocholanolone-G in vivo occurs by glucuronidation of one or more metabolites of testosterone rather than testosterone itself. Andros-G is also produced from the metabolism of DHT (Mauvais-Jarvis et al 1968), androsterone (Rosenfeld et al 1980), androstenedione and DHEA (Vande Wiele et al 1962), as well as DHEAS (Roberts et al 1961).

Fukushima et al (1954) showed that intravenous infusion of testosterone produced more urinary etiocholanolone than androsterone and that this production was maximized 5-9 hours following the infusion. Intramuscular administration of testosterone resulted in an even greater production of urinary etiocholanolone making the ratio of urinary androsterone to etiocholanolone even smaller. These data show that the androgenic actions of testosterone on various tissues can be controlled locally by changing the activity of either 5α or 5β reductase (since 5β metabolites are biologically inert). In addition, these data show that the overall metabolic pathways for the elimination of
testosterone are the same regardless of 5α or 5β reduction (since the major metabolite remains as either Andros-G or etiocholanolone-G).

The 5α- and 5β-reductases appear to undergo age related shifts in activity since the ratio of etiocholanolone-G to Andros-G (5β to 5α) decreases with age. Deslypere et al (1982) showed that this ratio dropped from a value of 1.63 in young men (18-22 years old) to 1.06 in elderly men (59-78 years old).

In summary, serum Andros-G levels are very high, exceeding those of Adiol-G. Andros-G is a product of testosterone as well as DHT metabolism and as such may be an important marker of androgen metabolism. There is not enough available information to determine whether serum levels of Andros-G would adequately reflect peripheral DHT metabolism.

IV.
SUMMARY

DHT stimulation of androgen dependent peripheral tissues is critical to the normal functioning of these tissues. Should DHT production be increased, or DHT elimination be decreased, hyperstimulation of these tissues would result. Such hyperstimulation could produce undesirable conditions such as benign prostatic hyperplasia
in men or hirsutism in women. Clinical treatment of these conditions could be initiated earlier in their development if a highly selective marker for peripheral DHT metabolism could be identified. Such early treatment would undoubtedly produce less suffering for the patient. Therefore, efforts have been made to identify a serum marker of DHT metabolism which is elevated above the normal range in all hyperandrogenic subjects.

The androgens testosterone and dihydrotestosterone can be produced from the metabolism of many steroids, including Adione, DHEA and DHEAS. These androgens can in turn be metabolized to many other steroids including Adiol and androsterone. Androgen glucuronides are also produced and are present in levels which exceed those of the unconjugates steroids.

While hirsutism is characterized by elevated serum levels of both the androgens and their precursors, these steroids are not elevated in all women within this group. As such, serum levels of these steroids fail to identify all members of the population. For this reason, other more specific markers have been sought. Several investigators have suggested that Adiol–G is a highly selective marker of hirsutism. Others have failed to support this conclusion. This thesis addresses the issue of whether androgen glucuronides are the best currently available markers of peripheral androgen metabolism.
THE EXPERIMENTS
I.

EXPERIMENTAL RATIONALES

The data presented in the literature review suggest that androgen glucuronides may be the best currently detectable serum markers of peripheral DHT metabolism. The data presented in this thesis address three different aspects of this relationship.

1) Is glucuronidation the initial step in the elimination of DHT, or is DHT first metabolized to Adiol, with the resulting Adiol then glucuronidated?

2) Is Adiol-G produced from the metabolism of DHT by peripheral tissues only?

3) Do the adrenal and ovary both contribute equally to the androgens which are ultimately metabolized to androgen glucuronides.
**EXPERIMENT J: Total Adiol-G and Adiol 3-G Levels in Serum of Normal Men and Women and in Normal Men Infused with $^3$H-DHT.**

By determining which isomeric form of Adiol-G is the major circulating form in serum, the pathway of DHT conversion to Adiol-G may be better understood. Glucuronidation of DHT followed by metabolism to Adiol-G would result in Adiol 17-G production only. However, if DHT is first converted to Adiol and the resulting Adiol is glucuronidated non-selectively at either available hydroxyl group, then Adiol 3-G and Adiol 17-G would be produced equally. For this reason the serum levels of Adiol-G isomers were determined in men and women.

Adiol-G is usually measured by hydrolyzing the Adiol-G molecule and then assaying the resulting unconjugated androstanediol in an androstanediol radioimmunoassay. This method of analysis fails to distinguish between the isomers and as such eliminates the information about isomer levels. For this reason, we have developed assays which allow for independent measurement of Adiol 3-G, Adiol 17-G and total Adiol-G.

In Experiment 1 Part A, baseline levels of total Adiol-G as well as Adiol 3-G were determined in both men and women. The Adiol 17-G assay was not available at the time
this work was done so these values were derived by subtracting Adiol 3-G levels from total Adiol-G levels. In this way the balance between Adiol 3-G and Adiol 17-G isomer production was determined. This indicated whether the two isomers were produced equally or if one isomer was preferentially produced. Subsequently, tritiated DHT was infused into normal male volunteers and the percentage of Adiol-G present as Adiol 3-G was determined. These results were compared to the baseline values measured previously. If the ratio of isomers produced was not the same in both experiments, then the possibility would exist that some precursor other than DHT was contributing significantly to Adiol-G production.

In Experiment 1 Part B, the levels of Adiol 3-G, Adiol 17-G and total Adiol-G were determined in normal men. Adiol 17-G was measured directly in this experiment using the newly developed Adiol 17-G assay. The sum of Adiol 3-G and Adiol 17-G levels were compared with the measured value of total Adiol-G. These data confirmed the results obtained in Part A of this experiment and in addition showed that the diglucuronide form of Adiol-G is not detectable in serum.
EXPERIMENT 2: Production of Adiol-G by Glucuronyl Transferase

In Experiment 2 Part A, the in vitro metabolism of Adiol to each Adiol-G isomer by glucuronyl transferase prepared from rat prostate was determined. Experiment 2 Part B examined the same process using glucuronyl transferase prepared from rat liver, while Experiment 2 Part C examined isomer production by human liver glucuronyl transferase. Prostate tissue was studied since this is an androgen (primarily DHT) dependent tissue and has been shown to produce androgen glucuronides from testosterone (see literature review). The liver was examined in order to determine whether Adiol-G can be produced from tissues other than peripheral androgen dependent tissues. Liver was selected since it is a major site for the conjugation of many other compounds.

The percentage of each Adiol-G isomer produced from the metabolism of Adiol by each tissue type was compared to serum Adiol 3-G and Adiol 17-G levels present in the rat or human. From this comparison, inferences are drawn regarding the contribution of the hepatic versus peripheral compartments to Adiol-G production in vivo.
EXPERIMENT 3: Androsterone Glucuronide as a Marker of Adrenal Hyperandrogenism

The results from Experiments 1 and 2 showed that Adiol 3-G is only a minor constituent in overall Adiol-G production. However, glucuronidation of the 3-hydroxyl group is an important step in androgen metabolism. As was described in the review of literature, one of the major products of androgen metabolism in vivo and in vitro is androsterone glucuronide (Andros-G); a 3-glucuronide. In Experiment 3, the source and control of Andros-G production was determined in normal and hyperandrogenic women. Since both Adiol-G and Andros-G have been shown to be produced from the metabolism of androgens, the usefulness of each in predicting the development of hirsutism in hyperandrogenic women was determined.
i) Androstanediol 3-glucuronide

Serum Adiol 3-G was measured directly (without hydrolysis) by radioimmunoassay (RIA) in ether-extracted serum after the androgen conjugates were separated by high performance liquid chromatography (HPLC).

Adiol 3-G standard was synthesized by Dr. P.N. Rao (1986). This preparation served as the assay standard. The antibody for the RIA was raised against Adiol 3-G by coupling Adiol 3-G to bovine serum albumin (BSA) through the free carboxyl group on the glucuronide moiety, using a carbodiimide condensation reaction (Abraham 1971). 75 μg of this antigen were used for immunization of rabbits, according to the method of Vaitukaitis et al. (1971). The cross-reactivity of the antiserum used in the RIA is shown in Table 1. Since the antibody cross-reacted with free Adiol, [9,11]^{3}H-Adiol (DuPont-NEN, Boston, MA) was used as the labeled ligand in the RIA while [9,11]^{3}H-Adiol 3-G was used for determination of procedural losses. This ^{3}H-Adiol 3-G was prepared by exposing the 9,11 unsaturated precursor
TABLE 1

Percent cross-reaction of antiserum against 5α-androstane-3α,17β-diol 3α-glucuronide-BSA (1:20,000 dilution, as used in the RIA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conjugated Steroids</strong></td>
<td></td>
</tr>
<tr>
<td>Androstanediol 3-Glucuronide</td>
<td>100.0</td>
</tr>
<tr>
<td>Androstanediol 17-Glucuronide</td>
<td>0.3</td>
</tr>
<tr>
<td>Androsterone Glucuronide</td>
<td>1.8</td>
</tr>
<tr>
<td>Dehydroepiandrosterone Sulfate</td>
<td>1.0</td>
</tr>
<tr>
<td>Dihydrotestosterone Glucuronide</td>
<td>0.3</td>
</tr>
<tr>
<td>Etiocholanolone Glucuronide</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Unconjugated Steroids</strong></td>
<td></td>
</tr>
<tr>
<td>Androstaneol</td>
<td>215.0</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>230.0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1.6</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.2</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>707.0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>183.0</td>
</tr>
</tbody>
</table>
(a triacetate methyl ester of 5α-androst-9,11-ene-3α, 17β-diol 3-glucuronide) to tritium gas (Rao 1986).

For serum sample preparation, 2,000 cpm of $^3$H-Adiol 3-G were added to 500 μL serum. Unconjugated steroids were extracted twice with 3 mL ether per extraction. The aqueous phase, containing the androgen conjugates, was applied to an equilibrated (10 mL methanol followed by 10 mL water) C18 sample preparatory column (Sep Pak, Waters Scientific, Mississauga, Ontario) to remove serum proteins. The column was washed with 20 mL water and the androgen conjugate fraction was eluted from the column in 3 mL methanol. The sample was evaporated to dryness under vacuum (Savant Speed Vac, Emerston Instruments, Scarborough, Ontario) and reconstituted in 100 μL methanol. Adiol 3-G was separated from other androgen conjugates by reverse phase HPLC (Rittmaster et al 1988c), using an isocratic mobile phase of 35:65 acetonitrile: 0.1% trifluoroacetic acid in water delivered at a flow rate of 1 mL/min. A 250 mm Alltech Adsorbosphere C18, 5 micron column (Alltech Applied Science, Deerfield, IL) was used for the chromatography. Retention times for the androgen conjugates using this separation procedure are given in Table 2. The fraction between minutes 9 and 11 was collected, evaporated to dryness under vacuum, and reconstituted in 600 μL of pH 7.5 phosphate buffered saline - 0.1% gelatin (assay buffer). 150 μL were taken for determination of procedural losses. For the
TABLE 2

Androgen conjugate retention times on HPLC using eluting solvent 35:65 acetonitrile:0.1% trifluoroacetic acid in water. Retention times were determined by ultraviolet spectrophotometry and verified by isotope elution profiles for those steroids for which tritiated isotopes were available.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroepiandrosterone Sulfate</td>
<td>3.9</td>
</tr>
<tr>
<td>Androsterone Sulfate</td>
<td>4.7</td>
</tr>
<tr>
<td>Testosterone Glucuronide</td>
<td>6.2</td>
</tr>
<tr>
<td>Dehydroepiandrosterone Glucuronide</td>
<td>6.4</td>
</tr>
<tr>
<td>Androstanediol 3-Glucuronide</td>
<td>9.7</td>
</tr>
<tr>
<td>Dihydrotestosterone Glucuronide</td>
<td>9.9</td>
</tr>
<tr>
<td>Androstanediol 17-Glucuronide</td>
<td>10.3</td>
</tr>
<tr>
<td>Etiocholanolone Glucuronide</td>
<td>15.0</td>
</tr>
<tr>
<td>Androsterone Glucuronide</td>
<td>16.5</td>
</tr>
</tbody>
</table>
assay, 200 µL sample were added to 100 µL of assay buffer. Assay standards were brought up in 300 µL assay buffer. 100 µL assay buffer containing 10,000 to 15,000 cpm of [9,11]-\(^3\)H-Adiol and 100 µL of a 1:4,000 dilution of antibody in RIA buffer (final tube dilution of 1:20,000) was added and the assay incubated at 4° C for 16 - 20 hrs. Unbound ligand was separated from bound ligand by dextran coated charcoal prepared as a 0.1% dextran (70,000 mol wt): 0.5% activated charcoal (neutralized) suspension in phosphate buffered saline, pH 7.4. Supernatant radioactivity was determined in an LKB model 1211 RackBeta scintillation counter following the addition of 5 mL of scintillation cocktail (Aquasol-2, Dupont-NEN). All samples were assayed in duplicate. The RIA data were analyzed by a computer program that used weighted regression of the logit-log-transformed data. The results were corrected for procedural losses.

**Assay Validation:** The dose range for the RIA was 0.026-1.71 pmol/tube (12-800 pg/tube), the mean half-maximal binding point was 0.39 ± 0.09 pmol/tube (184 ± 44 pg/tube), and the slope of the dose-response curve was -3.09 ± 0.16. The intra- and inter-assay coefficients of variation for the entire procedure were 10% and 17% respectively. The antibody bound negligible amounts of Adiol 17-G. The value in blank serum (obtained from a woman with gonadal
dysgenesis and Addison’s disease, in whom undetectable levels of serum testosterone, dihydrotestosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione were present) was less than 0.26 nmol/L. Dilutions of extracts from serum of normal men ran parallel to the standard curve and Adiol 3-G standard, when added to blank serum, was quantitatively recovered. When tritium-labeled unconjugated androgens were added to normal serum, and the serum processed as described for the RIA, none of the radioactivity appeared in the Adiol 3-G peak of the HPLC chromatogram. When $^3$H-Adiol 3-G was added to pooled male serum and the Adiol 3-G peak from the HPLC was divided into 15 second fractions, the immunoreactivity paralleled the radioactivity, indicating that the immunoreactivity in the serum sample eluted in a peak synchronous with that of $^3$H-Adiol 3-G (Figure 2).

ii) Androstanediol 17-Glucuronide

Serum Adiol 17-G was measured directly (without hydrolysis) by RIA in ether-extracted serum after the androgen conjugates were separated by high performance liquid chromatography.

Sample preparation was identical to the procedure described for Adiol 3-G except that $^3$H-Adiol 17-G (produced
[9,11]-^3H ANDROSTANEDIOL 3-GLUCURONIDE WAS ADDED TO NORMAL MALE SERUM AND THE ELUTION PROFILES OF IMMUNOLOGIC ACTIVITY (BY RIA) AND RADIOACTIVITY FOLLOWING HPLC SEPARATION (0.25 MIN/FRACTION) WAS DETERMINED. THE BOTTOM PANEL SHOWS THE IMMUNOLOGIC ELUTION PROFILE EXPRESSED AS PERCENT OF TOTAL IMMUNOLOGIC ACTIVITY. THE TOP PANEL SHOWS THE ELUTION PROFILE OF [9,11]-^3H ANDROSTANEDIOL 3-GLUCURONIDE EXPRESSED AS PERCENT OF TOTAL ACTIVITY ELUTING FROM THE HPLC.
ADIOL 3-G ELUTION PROFILE (% TOTAL)

Immunoactivity

Radioactivity

FRACTION

4
8
12

FIGURE 2
by the procedure of Rao 1986), rather than $^3$H-Adiol 3-G, was used to determine procedural losses. Using the same HPLC separation conditions as described for Adiol 3-G, Adiol 17-G eluted between minutes 9-12. This peak was collected, dried under vacuum and reconstituted in 600 $\mu$L pH 7.5 assay buffer (same buffer as described for Adiol 3-G assay). 150 $\mu$L were taken for determination of procedural losses.

$^3$H-Adiol 17-G was used as labeled ligand in the RIA. An antibody raised against Adiol 17-G-BSA was developed by Dr. P. N. Rao according to his previously published procedure (Rao 1984), with the exception that Freund's incomplete adjuvant was used in the preparation of the immunogen. Cross reactivity of other steroids and steroid conjugates with this antibody are given in Table 3.

For the assay, 200 $\mu$L of sample were added to 100 $\mu$L of assay buffer. Adiol 17-G (courtesy of Dr P.N. Rao) was used as assay standard. Each dilution of standard was added in 300 $\mu$L assay buffer. 100 $\mu$L of assay buffer containing 8,000 - 10,000 cpr $^3$H-Adiol 17-G and 100 $\mu$L of a 1:2,000 dilution of antibody (final tube dilution of 1:10,000) were then added. The assay was left to incubate at 4° C for 16-20 hours. Bound radioactivity was measured and analyzed as described for the Adiol 3-G assay.

**Assay Validation:** The dose range for the RIA was 0.0667 to 2.134 pmol/tube (31.25 to 1000 pg/tube). The mean half
TABLE 3

Percent cross-reaction of antiserum against 5α-androstane-3α,17β-diol 17β-glucuronide-BSA (1:10,000 dilution, as used in the RIA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstanediol 17-Glucuronide</td>
<td>100</td>
</tr>
<tr>
<td>Androstanediol</td>
<td>11</td>
</tr>
<tr>
<td>Androsterone</td>
<td>7</td>
</tr>
<tr>
<td>Androstanediol 3-Glucuronide</td>
<td>6</td>
</tr>
<tr>
<td>Dihydrotestosterone Glucuronide</td>
<td>2</td>
</tr>
<tr>
<td>Testosterone Glucuronide</td>
<td>0</td>
</tr>
<tr>
<td>Dihydrotestosterone (5α &amp; 5β)</td>
<td>0</td>
</tr>
<tr>
<td>Androsterone Glucuronide</td>
<td>0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>0</td>
</tr>
<tr>
<td>Androstanedione</td>
<td>0</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0</td>
</tr>
</tbody>
</table>
maximal binding point was 0.327 ± 0.028 pmole/tube. The slope of the dose-response curve was -2.2527 ± 0.129 and the intra- and inter-assay coefficients of variation were 3% and 8% respectively. The assay blank (using the same serum as described for the Adiol 3-G assay) was 0.13 ± 0.07 nmole/L. Dilutions of extracts of serum from normal men ran parallel to the standard curve and Adiol 17-G standard, when added to blank serum, was quantitatively recovered. When tritium-labeled unconjugated androgens were added to normal serum, and the serum processed as described for the RIA, none of the radioactivity appeared in the Adiol 17-G peak from the HPLC separation. When \(^3\)H-Adiol 17-G was added to pooled male serum and the Adiol 17-G peak from the HPLC was divided into 15 second fractions, the immunoreactivity paralleled the radioactivity, indicating that the immunoreactivity in the serum sample eluted in a peak synchronous with that of the \(^3\)H-Adiol 17-G (Figure 3).

iii) Total Androstanediol Glucuronide

Total Adiol-G levels were determined by removal of the glucuronide moiety from the steroid nucleus by enzymatic hydrolysis followed by purification and assay of the liberated Adiol.

2000 cpm of \(^3\)H-Adiol 17-G were added to 500 µL of serum for determination of procedural losses. The unconjugated
FIGURE 3

HPLC ELUTION PROFILE OF ANDROSTANEDIOL 17-GLUCURONIDE

[9,11]-3H ANDROSTANEDIOL 17-GLUCURONIDE WAS ADDED TO NORMAL MALE SERUM AND THE ELUTION PROFILES OF IMMUNOLOGIC ACTIVITY (BY RIA) AND RADIOACTIVITY FOLLOWING HPLC SEPARATION (0.25 MIN/FRACTION) WAS DETERMINED. THE BOTTOM PANEL SHOWS THE IMMUNOLOGIC ELUTION PROFILE EXPRESSED AS PERCENT OF TOTAL IMMUNOLOGIC ACTIVITY. THE TOP PANEL SHOWS THE ELUTION PROFILE OF [9,11]-3H ANDROSTANEDIOL 17-GLUCURONIDE EXPRESSED AS PERCENT OF TOTAL ACTIVITY ELUTING FROM THE HPLC.
steroids were removed by extraction with ether (2 extractions of 3.5 mL ether per extraction). The aqueous phase containing the androgen conjugate fraction was adjusted to a pH value of 5.0 with 0.2 M acetic acid. Hydrolysis was performed at 45° C for 18 hrs following the addition of 20,000 units of β-glucuronidase in 100 μL of 0.1 M sodium acetate buffer (pH 5). The liberated steroids were extracted with ether (2 extractions of 4 mL ether per extraction), dried under a stream of nitrogen and brought up in 200 μL 70% iso-octane: 30% benzene and added to ethylene glycol:celite columns (0.75 mL ethylene glycol / 1.5 g baked celite). The columns were washed with 12 mL of 70% iso-octane: 30% benzene. The Adiol fraction was then eluted with 7 mL of 40% iso-octane: 60% benzene. Under these elution conditions, androsterone, DHT and testosterone all elute in the first 12 mL wash. Of these three unconjugated steroids, testosterone elutes closest to the Adiol peak. The separation between testosterone and Adiol is shown in Figure 4. When tritiated androsterone, DHT and testosterone (50,000 cpm total counts) were added to a celite column and eluted under the above conditions, less than 1% of added counts were present in the Adiol fraction.

Following collection, the Adiol peak was evaporated to dryness under a stream of nitrogen and the sample reconstituted in 600 μL assay buffer. 150 μL of the sample were taken for recovery determination and 200 μL aliquots
FIGURE 4

SEPARATION OF UNCONJUGATED TESTOSTERONE AND ANDROSTANEDIOL BY CELITE CHROMATOGRAPHY

Androstanediol

Testosterone

% TOTAL ACTIVITY

FRACTION

FIGURE 4
were taken for RIA analysis. The samples were assayed in duplicate. The RIA procedure was identical to that for Adiol 17-G with the exception that the antibody used was the same as that used in the Adiol 3-G assay (due to its high cross reactivity with free Adiol).

**Assay Validation:** The dose range for the RIA was 0.066–2.13 pmol/tube (31–1000 pg/tube). The mean half maximal binding point was $0.48 \pm 0.01$ pmol/tube, and the slope of the dose-response curve was $-3.36 \pm 0.06$. The intra- and interassay coefficients of variation for the entire procedure were 6.2% and 9.8% respectively. Serial dilutions of Adiol 3-G standard, when added to blank serum, hydrolyzed and purified by celite chromatography, were quantitatively recovered in the Adiol assay, indicating that the Adiol and Adiol 3-G standards were equivalent. The value in blank serum was less than 0.30 nmol/L. Serial dilutions prepared from the serum of normal men ran parallel to the standard curve.

The Adiol standard used by Nichols Institute (Los Angeles, Ca) was 24% more potent than our standard when measured in our assay (the standard curves were parallel). When these correction factors were used, levels of Adiol-G in aliquots of normal male serum were similar in both assays.
iv) Androsterone Glucuronide

Serum Andros-G was measured directly (without hydrolysis) by RIA in ether-extracted serum after the androgen conjugates were separated by HPLC.

[9,11]-3H-Andros-G tracer, used for both recovery determination and the labeled ligand in the RIA, was prepared from [9,11]-3H-androsterone (2.0 TBq/mmol; New England Nuclear, Boston, mA) using rat-liver glucuronyl transferase with uridine diphosphoglucuronic acid (UDPGA) as cofactor. 2 μCi [9,11]-3H-androsterone in 50 μL methanol was added to 5 mg homogenized rat liver (from a 250 g male Sprague-Dawley rat; Canadian Hybrid Farms, Hall Harbour, NS) in 950 μL pH 7.4 Hanks Balanced Salt Solution containing 25 mmol/L HEPES buffer and 5 mmol/L UDPGA. The incubation was carried out at 37° C for 2 hrs followed by centrifugation at 20,000 g for 1 hour. Unreacted [9,11]-3H-androsterone was removed from the supernatant by extraction with ether. [9,11]-3H-Andros-G was purified as described below for serum samples.

For serum sample preparation, 2000 cpm 3H-Andros-G was added to 500 μL serum. Unconjugated steroids were removed by extraction with ether (3.5 mL par extraction) and the androgen conjugate fraction was prepared as described for Adiol 3-G. Andros-G was separated from other androgen
conjugates by reverse phase HPLC using an isocratic mobile phase of 40:60 acetonitrile:0.1% trifluoroacetic acid in water. Andros-G eluted as a single peak with a retention time of 9-11 minutes. Retention times for other androgen conjugates are given in Table 4. In Experiment 1 Part B, the Andros-G peak eluted between minutes 15 and 17 using an isocratic mobile phase of 35:65 acetonitrile:0.1% trifluoroacetic acid in water (the elution conditions used for Adiol-G collection). Following chromatography, the appropriate fractions were collected, evaporated to dryness, and reconstituted in 500 μL pH 7.5 phosphate buffered saline – 0.1% gelatin (assay buffer). 200 μL of sample were taken for determination of procedural losses. For the RIA, 50 μL sample were added to 250 μL assay buffer. Assay standards were brought up in 300 μL assay buffer. 100 μL assay buffer containing 9,000-10,000 cpm ³H-Andros-G and 100 μL of a 1:840 dilution of antibody (raised against Adiol 3-G as described for Adiol 3-G assay: final tube dilution of 1:4200) was added and the assay incubated at 4° C for 16-20 hrs. Unbound ligand was removed and bound ligand was measured and analyzed as described for the Adiol 3-G assay.

Assay Validation: The dose range for the RIA was 0.07 to 8.8 pmol/tube (32 to 4096 pg/tube), the mean half-maximal binding point was 0.54 ± 0.07 pmol/tube, and the slope of the dose response curve was -1.9 ± 0.1. The intra-assay
TABLE 4

Androgen conjugate retention times on HPLC using eluting solvent 40:60 acetonitrile:0.1% trifluoroacetic acid in water. Retention times were determined by ultraviolet spectrophotometry and verified by isotope elution profiles for those steroids for which tritiated isotopes were available.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroepiandrosterone Sulfate</td>
<td>4.2</td>
</tr>
<tr>
<td>Androsterone Sulfate</td>
<td>4.5</td>
</tr>
<tr>
<td>Testosterone Glucuronide</td>
<td>5.6</td>
</tr>
<tr>
<td>Dehydroepiandrosterone Glucuronide</td>
<td>6.0</td>
</tr>
<tr>
<td>Epiandrosterone Glucuronide</td>
<td>6.5</td>
</tr>
<tr>
<td>Dihydrotestosterone Glucuronide</td>
<td>6.9</td>
</tr>
<tr>
<td>Androstanediol 17-Glucuronide</td>
<td>7.6</td>
</tr>
<tr>
<td>Etiocholanolone Glucuronide</td>
<td>8.4</td>
</tr>
<tr>
<td>Androsterone Glucuronide</td>
<td>9.7</td>
</tr>
</tbody>
</table>
coefficients of variation are less than 6% at all points on the standard curve. The inter-assay coefficient of variation, measured in pooled serum in 20 assays, was 10%. Serial dilutions of serum extracts from normal men were parallel to the standard curve. Andros-G standard, when added to blank serum (see Adiol 3-G), was quantitatively recovered, while the blank serum itself was 4.1 ± 0.9 (SEM) nmol/L. When tritium-labeled unconjugated androgens were added to normal serum and the serum processed as described above, none of the radioactivity appeared in the Andros-G peak of the HPLC chromatogram. When $^3$H-Andros-G was added to pooled male serum, and the Andros-G peak from the HPLC was divided into 15 second fractions, the immunoactivity paralleled the radioactivity, indicating that the immunoactivity in the serum sample eluted in a peak synchronous with that of the $^3$H-Andros-G (Figure 5). As was noted in the literature review, the 5β-isomer of androsterone (etiocholanolone) is present in serum in high levels. Therefore, it is quite likely that the 5β-isomer of Andros-G (Etiocholanolone-G) would also be elevated in serum. While our chromatography system separates these two isomers, the actual peaks elute within two minutes of each other. For this reason it was necessary to show that Etiocholanolone-G was not being detected in our assay. To demonstrate this, the Andros-G peak from pooled serum was
FIGURE 5

HPLC ELUTION PROFILE OF ANDROSTERONE-GLUCURONIDE

[9,11]-^3H ANDROSTERONE-GLUCURONIDE WAS ADDED TO NORMAL MALE SERUM AND THE ELUTION PROFILES OF IMMUNOLOGIC ACTIVITY (BY RIA) AND RADIOACTIVITY FOLLOWING HPLC SEPARATION (0.25 MIN/FRACTION) WERE DETERMINED. THE BOTTOM PANEL SHOWS THE IMMUNOLOGIC ELUTION PROFILE EXPRESSED AS PERCENT OF TOTAL IMMUNOLOGIC ACTIVITY. THE TOP PANEL SHOWS THE ELUTION PROFILE OF [9,11]-^3H ANDROSTERONE-GLUCURONIDE EXPRESSED AS PERCENT OF TOTAL ACTIVITY ELUTING FROM THE HPLC.
ANDROS-G ELUTION PROFILE (% TOTAL)

Immuoactivity

Radioactivity

0 10 20 30

4 8 12

FRACTION

FIGURE 5
hydrolyzed with β-glucuronidase, re-chromatographed using Celite chromatography and reassayed in an RIA for androsterone using an antibody that did not cross-react with etiocholanolone. The recovery-corrected values for Andros-G as determined by this hydrolysis method were compared to the levels obtained by the direct assay. Andros-G levels in 5 normal women were not statistically different when measured in the direct assay (108 ± 31 nmol/L) and compared to the hydrolysis assay (133 ± 15 nmol/L).
II.

GENERAL METHODS: PART B

BINDING ASSAY

In Experiment 1, Part A and Experiment 2, Parts A, B and C, Adiol 3-G was separated from Adiol 17-G by precipitation using an antibody specific for Adiol 3-G. Since binding of the Adiol 3-G could only be maximized by using a high antibody titer, the non-specific binding which resulted from this high titer had to be eliminated. For this reason the IgG fraction from the antiserum was purified and used in the binding assay.

Preparation of IgG Fraction: Protein-A-Sepharose CL-4B (1.5 g; Pharmacia Fine Chemicals, Piscataway, NJ) was swollen in buffer A (10 mmol/L Na$_2$HPO$_4$ and 120 mmol/L NaCl, pH 8) for 15 hours, then washed with an additional 700 mL buffer A and placed in a 5 mL column. 2 mL of antibody were diluted to 10 mL with buffer A and loaded onto the column. The column was then washed sequentially with 30 mL each of the following buffers: 1) buffer A; 2) 0.1 mol/L sodium acetate buffer, pH 6; and 3) 0.1 mol/L sodium acetate buffer, pH 4. 3 mL aliquots were collected. The antibody eluted from the column during the third and fourth fractions of the pH 4 wash. The pH of the antibody solution was adjusted to 7.5 with 1.7 mol/L Na$_2$HPO$_4$ before use in the assay.
For the binding assay, 100 µL undiluted purified antibody were added to 100 µL sample and 300 µL RIA buffer. The remainder of the binding assay procedure was identical to that described above for the Adiol 3-G assay.

Binding Assay Validation: When a purified immunoglobulin preparation of normal rabbit serum was used instead of the purified Adiol 3-G antibody and 800 cpm $^3$H-Adiol 3-G or $^3$H-Adiol 17-G were added, 1.8 ± 0.5% was bound. This nonspecific binding did not change when excess unlabeled Adiol 3-G was added. Since this assay was intended to be used in experiments where there could be substantial levels of unlabeled Adiol 3-G interfering in the binding of $^3$H-Adiol 3-G, the ability of the purified IgG fraction to bind $^3$H-Adiol 3-G in the presence of varying amounts of unlabeled compound was determined. This calibration experiment was done using a range of unlabeled Adiol 3-G which exceeded the range in serum by more than a factor of 10. The Adiol 3-G was added to 800 cpm $^3$H-Adiol 3-G. Under these conditions, the antibody bound 80 ± 1% of the radioactivity, independent of the amount of unlabeled Adiol 3-G added. When 800 cpm $^3$H-Adiol 3-G were added to 3.5 mL plasma from a normal man (the amount of plasma extracted in Experiment 1 Part B), 80% of the counts were bound by the purified antibody. Similarly, the purified IgG specifically bound 8.4% of the $^3$H-Adiol 17-G.
II.

GENERAL METHODS: PART C

TISSUE PREPARATION AND INCUBATION

Rat ventral prostate tissue was dispersed in 25 mM HEPES-Hanks (Hanks' balanced salt solution, containing 25 mM HEPES buffer, pH 7.4) containing 0.25% collagenase and 0.005% deoxyribonuclease. Cell dispersion was conducted in a 37°C shaking water bath for 45 min. The cells were then centrifuged for 4 min at 400 x g, the supernatant was removed and the cells were resuspended in 5 mL HEPES-Hanks and counted in a hemocytometer. The cells were recentrifuged, resuspended in 1 mL HEPES-Hanks, and disrupted by sonication (Sonifier Cell Disrupter, Heat Systems-Ultrasonics, Inc., Plainview, NY). A sufficient amount of this suspension was then added to each enzyme assay tube to provide 2 x 10^6 cell equivalents/tube. For the enzyme assay, 1 μCi [3H]-steroid was added to 2 x 10^6 cell equivalents and incubated at 37°C for 90 min in 1 mL HEPES-Hanks, pH 7.4, containing 5 mM UDP-glucuronic acid. All experimental comparisons were performed on the same day using the same cell suspensions.

Rat liver and human liver tissue was minced and frozen in 100 to 200 mg aliquots at -80°C. At time of assay, an aliquot of minced tissue was weighed and then homogenized with a Brinkmann Polytron (PT 10/35 with a PTA 10S probe;
Brinkmann Instruments Westbury, NY) in HEPES-Hanks. The homogenates were then diluted in HEPES-Hanks to a final concentration of 0.5 mg/500 uL. For each assay, 0.5 μCi [3H]-steroid was added to 500 μL enzyme preparation and incubated at 37° C for 30 min in 1 mL total volume of pH 7.4 HEPES-Hanks, containing 5 mM UDP-glucuronic acid (Green 1985). All enzyme assays were done in duplicate unless otherwise stated. All experimental comparisons were performed on the same day using the same enzyme preparation.

The enzyme reactions were stopped by extraction of the remaining unconjugated steroids with ether. The aqueous phase was centrifuged at 20,000 X g for 60 minutes. The ether phase was dried under vacuum and then reconstituted in 100 μL benzene-methanol (9:1) containing 0.35 M unlabeled steroids (testosterone, DHT, Adiol, androsterone and androstenedione). These unconjugated steroids were separated by thin layer chromatography (TLC), using high resolution silica gel G plates (Applied Science Laboratory, Deerfield, IL) and were eluted twice with benzene-acetone (4:1). Radioactive zones were located and integrated with a System 200 Imaging Scanner (Bioscan, Washington, D.C.).

Steroid conjugates in the aqueous phase were separated by HPLC as described for Adiol 3-G (General Methods, Section A-i, pg 75). The enzyme assay for rat prostate and rat liver glucuronyl transferase was developed and validated by
C. Leopold (Rittmaster 1988d). The enzyme assay for human liver glucuronyl transferase was developed and validated by H. Zwicker (to be published).

Data obtained in the calibration studies showed that the glucuronyl transferase enzyme preparations obtained from rat liver and rat prostate both had a pH maximum of 7.4 and Km value of 15 μM. The Vmax value obtained from rat liver was 800 times greater than was that obtained from rat prostate.
III.

EXPERIMENT 1  PART A

TOTAL ANDROSTANEDIOL-GLUCURONIDE AND
ANDROSTANEDIOL 3-GLUCURONIDE LEVELS IN SERUM
OF NORMAL MEN AND WOMEN AND IN NORMAL MEN INFUSED WITH
$^3$H-DIHYDROTESTOSTERONE
INTRODUCTION

To determine which isomer of Adiol-G is the major circulating form of this androgen conjugate, total Adiol-G and Adiol 3-G levels were measured in normal men and normal women. To determine which isomer is the predominant physiological DHT metabolite, normal men were infused with $^3$H-DHT and the percentage of cpm present as Adiol 3-G was compared to the cpm present as total Adiol-G.

METHODS

Baseline Levels: The subjects in this study included 10 normal women and 10 normal men, aged 21-45 years, taking no medications. The women had regular menses, had no evidence of acne or hirsutism and were sampled in the early follicular phase of the menstrual cycle. All serum samples were obtained between 1100 and 1600 h. Adiol 3-G and total Adiol-G were measured as described in General Methods, Sections A-i and -iii, pgs 75 & 85.

$^3$H-DHT Infusions: Four normal male volunteers were infused with [1,2]$^3$H-DHT (New England Nuclear Corp., Boston, MA) dissolved in 8% ethanol in saline. The infusion procedure was as previously described (Moghissi 1984). The serum was kindly supplied by Dr R. Horton.
3.5 mL serum were aliquoted per sample. Following extraction, the samples were run over C18 sample preparatory columns (General Methods, Section A-i, pg 75). The androgen conjugate fraction for each sample was collected and the individual components of the fraction were separated by HPLC. The fractions containing Adiol 3-G and Adiol 17-G were collected, pooled and dried under vacuum. Each sample was then reconstituted in 150 μL RIA buffer. 50 μL were used to determine the total counts in the Adiol-G fraction and the remaining 100 μL were used in the binding assay.

Statistics

All results are expressed as the mean ± SEM.

RESULTS

Baseline Levels

The mean serum Adiol 3-G and total Adiol-G concentration in the 10 normal men were 4.44 ± 0.49 nmol/L and 27.9 ± 2.8 nmol/L respectively. Adiol 3-G was 13.9 ± 3% of total Adiol-G in men.

The mean serum Adiol 3-G and total Adiol-G levels in 10 normal women were 2.64 ± 0.64 nmol/L and 14.9 ± 1.5 nmol/L. Adiol 3-G was 17.4 ± 3.6% of total Adiol-G in women.
There was no significant difference between men and women in the percentage of total Adiol-G present as Adiol 3-G (P>0.05, by Student's t-test).

**DHT Infusion**

When 800 cpm $^3$H-Adiol 3-G were added to 3.5 mL plasma from a normal man (the amount of plasma extracted from $^3$H-DHT infused men), 80% of the counts were bound by the purified antibody. Similarly, the purified IgG specifically bound 8.4% of the $^3$H-Adiol 17-G. Using these data, the relative amounts of $^3$H-Adiol 3-G and $^3$H-Adiol 17-G were determined using two equations; 1) $x + y = \text{total counts available for binding}$, and 2) $0.8x + 0.084y = \text{counts bound by the purified IgG}$, where $x$ is the counts/minute (cpm) for Adiol 3-G and $y$ is the cpm for Adiol 17-G, present in the Adiol-G peak.

Because DHT-G coeluted with Adiol-G in our HPLC system, any $^3$H-DHT-G present in the serum of the $^3$H-DHT-infused normal men would be included as total Adiol-G counts and could potentially be bound by the IgG (though the cross reactivity with the Adiol 3-G antiserum is very low, the binding assay used the IgG at a very high titer which would have increased the amount of DHT-G bound). To prevent such overestimates of Adiol-G counts, pooled plasma from $^3$H-DHT-infused normal men was extracted, and the glucuronides
were separated by HPLC, as described above. The Adiol-G fraction (9-11 min) was then hydrolyzed with β-glucuronidase (General Methods, Section A-iii, pg 85) and the resulting free steroids were purified by Celite chromatography to separate DHT from Adiol. Eight percent of the radioactivity eluted in the DHT fraction; the remainder eluted in the Adiol fraction. The appropriate corrections were made.

When the plasma from the 4 men infused with labeled DHT was extracted and added to the binding assay using the purified antibody, 14 ± 2% of the radioactivity attributable to Adiol-G was bound. Given that the IgG preparation bound 80% of the Adiol 3-G counts and 8.4% of the Adiol 17-G counts, then 17.4 ± 3.4% (Table 5) of the $^3$H-Adiol-G derived from $^3$H-DHT was $^3$H-Adiol 3-G (see Appendix A for example of calculations).

In one subject, the fractions eluting before and after the Adiol-G peak were examined for radioactivity and three additional peaks were detected. Two peaks eluted before Adiol-G, the first having a retention time of 2-3 minutes (27% of all counts present in the androgen conjugate peaks) and the second having a retention time of 5-6 minutes (9% of all counts present in the peaks). Since these peaks have retention times consistent with androgen sulfates (from
TABLE 5

Percentage of $^3$H-Adiol G present as $^3$H-Adiol 3-G in serum of normal men following infusion of [9,11]-$^3$H-DHT. A binding assay using purified Adiol 3-G antibody was used to determine counts in the Adiol-G peak present as Adiol 3-G. Sample calculations are shown in Appendix A.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Total Adiol G Counts</th>
<th>Counts Bound by the purified Adiol 3-G AB</th>
<th>% Counts as Adiol 3-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>585</td>
<td>157</td>
<td>25.8</td>
</tr>
<tr>
<td>2</td>
<td>662</td>
<td>143</td>
<td>19.7</td>
</tr>
<tr>
<td>3</td>
<td>765</td>
<td>131</td>
<td>13.1</td>
</tr>
<tr>
<td>4</td>
<td>723</td>
<td>112</td>
<td>10.8</td>
</tr>
<tr>
<td>$\bar{X}$</td>
<td></td>
<td>17.4</td>
<td>$\pm$ 3.4</td>
</tr>
</tbody>
</table>

$^3$H-Adiol G present as $^3$H-Adiol 3-G in serum of normal men following infusion of [9,11]-$^3$H-DHT. A binding assay using purified Adiol 3-G antibody was used to determine counts in the Adiol-G peak present as Adiol 3-G. Sample calculations are shown in Appendix A.
HPLC calibration studies), and the peaks were not hydrolyzed by β-glucuronidase, they are likely androgen-sulfates. The remaining additional peak of activity was found to elute at a time which corresponded to Andros-G. This peak contained 21% of the counts eluting in the androgen conjugate peaks. The Adiol-G peak was the largest peak, containing 43% of the activity eluting from the column.

SUMMARY

Adiol 3-G is only a minor constituent of total Adiol-G in both normal men and normal women. This same basic pattern was seen following 3H-DHT infusion in normal men. These data when taken together show that the major product of DHT metabolism is Adiol 17-G. The production of Andros-G from DHT may also be an important pathway of androgen metabolism.

These data are consistent with a model of DHT metabolism whereby DHT is glucuronidated directly and the resulting DHT-G is then metabolized to Adiol-G.
III.

EXPERIMENT 1  PART B

ANDROSTANEDIOL 3-GLUCURONIDE AND
ANDROSTANEDIOL 17-GLUCURONIDE LEVELS
IN SERUM OF NORMAL MEN
INTRODUCTION

Adiol 17-G levels were measured in men following the development of a specific RIA for Adiol 17-G. Total Adiol-G and Adiol 3-G levels were also determined and the percent of total Adiol-G present as Adiol 17-G was calculated. For each subject, the sum of the two isomers was compared to the measured total. This comparison was used to determine if some additional form of Adiol-G (such as Adiol-3,17-diglucuronide) was being measured in the total Adiol-G assay.

METHODS

Subjects: Total Adiol-G, Adiol 3-G and Adiol 17-G levels were independently measured in the same serum samples from 8 normal men aged 21-45 years.

Sample Analysis: The samples were analyzed for total Adiol-G, Adiol 3-G and Adiol 17-G as described in General Methods, Sections A-i, -ii and -iii, pages 75, 80 and 85. The Adiol 3-G and Adiol 17-G assays were calibrated against the total Adiol-G assay by hydrolyzing Adiol 3-G and Adiol 17-G standards and then measuring the liberated free Adiol in the total Adiol-G assay.
Statistics: The measured total Adiol-G values and the sum of the two isomers were compared for each subject by paired t-test analysis. All values are presented as mean ± SD.

RESULTS

Adiol 17-G standard was 106 ± 2% and Adiol 3-G was 99 ± 3% of their actual values when hydrolyzed and measured in the Adiol assay. The measured levels in both the Adiol 3-G and Adiol 17-G assays were adjusted by these correction factors to allow for direct comparison between assays.

The values for Adiol 17-G, Adiol 3-G and total Adiol-G are shown in Table 6. Adiol 3-G levels were 3.27 ± 1.86 nmol/L. Adiol 17-G levels were 12.97 ± 5.03 nmol/L. The sum of Adiol 3-G and Adiol 17-G levels for each individual averaged 16.24 ± 5.34 nmol/L while the average value of total Adiol-G measured by the total Adiol-G assay was 16.61 ± 5.24 nmol/L. There was no significant difference between the measured total Adiol-G value and the value derived from the summation of the Adiol 3-G and Adiol 17-G levels (P=0.513). Adiol 17-G was the predominant isomer of Adiol-G, being 77% of total.
TABLE 6

Adiol 3-G, Adiol 17-G and Total Adiol-G levels in serum of normal men as determined by radioimmunoassay. The sum of adiol 3-G and Adiol 17-G is compared to the measured value for Total Adiol-G. The percent of Total Adiol-G (measured) present as Adiol 17-G is presented.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Adiol 3-G (nmol/L)</th>
<th>Adiol 17-G (nmol/L)</th>
<th>Sum (nmol/L)</th>
<th>Total Adiol-G by RIA (nmol/L)</th>
<th>% of Total Adiol-G as Adiol 17-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>10.3</td>
<td>13.8</td>
<td>14.5</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
<td>11.4</td>
<td>14.5</td>
<td>15.5</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>2.8</td>
<td>13.7</td>
<td>16.5</td>
<td>18.5</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>2.8</td>
<td>10.7</td>
<td>13.5</td>
<td>12.1</td>
<td>89</td>
</tr>
<tr>
<td>5</td>
<td>2.7</td>
<td>20.6</td>
<td>23.3</td>
<td>24.1</td>
<td>86</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>18.8</td>
<td>20.7</td>
<td>18.8</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>4.8</td>
<td>6.6</td>
<td>7.6</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>7.6</td>
<td>13.2</td>
<td>20.8</td>
<td>20.7</td>
<td>64</td>
</tr>
</tbody>
</table>

X ± S.D. 3.3 ± 1.8 12.9 ± 5 16.2 ± 5.3 16.5 ± 5.2 77 ± 13

Paired t-test comparison between sum and measured total: P=0.515
SUMMARY

Direct measurement of Adiol 17-G levels has verified the previous conclusion that Adiol 17-G is the predominant isomer of Adiol-G in serum. In addition, the sum of Adiol 3-G and Adiol 17-G equalled the measured level of total Adiol-G, showing that Adiol di-glucuronide is either not present in serum or is present in relatively low amounts.

The results from both Part A and Part B of Experiment 1 suggest that DHT is glucuronidated and the resulting DHT-G is subsequently metabolized to Adiol 17-G.

It is also possible, however, that DHT is metabolized to Adiol and the resulting Adiol is then selectively glucuronidated at the 17-carbon position.

This is examined in Experiment 2.
IV.

EXPERIMENT 2  PART A

PRODUCTION OF ANDROSTANEDIOL GLUCURONIDE
BY GLUCURONYL TRANSFERASE
PREPARED FROM DISPERSED RAT PROSTATE CELLS
INTRODUCTION

The glucuronidation of androgens is catalyzed by the enzyme glucuronyl transferase. There are multiple forms of glucuronyl transferase, conjugating substrates at either the 3-carbon or 17-carbon positions. It is not known which form of this enzyme catalyzes the production of Adiol-G from Adiol. If the enzyme selectively glucuronidates the 17-carbon position, then Adiol should be converted to Adiol 17-G. If the enzyme favours the 3-carbon position, then Adiol would be metabolized to the Adiol 3-G isomer. In addition, substrate specificity of this enzyme is not known. If the enzyme is specific only for a given hydroxyl group, rather than the overall structure of the substrate molecule, then DHT would be as good a substrate for a 17-carbon enzyme and androsterone would be as good a substrate for a 3-carbon enzyme, as Adiol would.

To determine whether Adiol is preferentially metabolized at one of the two hydroxyl groups available for conjugation and whether the enzyme responsible for this metabolism is specific for Adiol, $^3$H-Adiol, $^3$H-DHT and $^3$H-androsterone were incubated with glucuronyl transferase prepared from rat prostate. The products of these incubations were identified.
METHODS

Tissue was prepared, incubations were performed and the samples processed as described in General Methods, Section C, page 101. The substrates used in the glucuronyl transferase enzyme assay were [9,11]-$^{3}$H-Adiol (specific activity, 2.0 TBq/mmol), [1,2]-$^{3}$H-DHT (1.9 TBq/mmol) and [9,11]-$^{3}$H-androsterone (2.0 TBq/mmol), obtained from DuPont-New England Nuclear (Boston, MA). $^{3}$H-Adiol 3-G, $^{3}$H-Adiol 17-G and $^{3}$H-Andros-G were obtained as described in General Methods, Sections A-i,-ii and -iv, pages 75, 80 and 92. $^{3}$H-DHT-G was produced by Dr. P. N. Rao by the methodology described for synthesis of $^{3}$H-Adiol 17-G (Rao 1986). Unlabeled Adiol, DHT and androsterone was obtained from Sigma Chemical Co. (St. Louis, Mo).

SAMPLE ANALYSIS: At the time that these experiments were performed, the Adiol 17-G assay had not yet been developed. For this reason, the levels of Adiol 3-G and Adiol 17-G produced during these incubations could not be determined directly using the appropriate RIA's. Instead, the Adiol-G isomers produced during these incubation were resolved by: 1) collecting 15-second fractions from the HPLC and analyzing the radioactive elution profile of the Adiol-G peaks; 2) using the Adiol 3-G binding assay to determine the percentage of Adiol-G counts present as Adiol 3-G.
**Method #1**: Calibration studies were run to determine the exact HPLC elution profiles of $^3$H-Adiol 3-G and $^3$H-Adiol 17-G. At the time that the samples were run for this experiment, 22% of Adiol 3-G counts eluted between 10.75 and 11.75 min, while 66% of Adiol 17-G counts eluted over this same time interval. The percentages of the two Adiol-G isomers were determined by solving the following equations:

1. $X + Y = \text{total counts in Adiol-G fraction}$;
2. $0.22X + 0.66Y = \text{counts collected between 10.75 and 11.75 minutes}$;

where $X$ represents the counts attributable to Adiol 3-G and $Y$ represents the counts attributable to Adiol 17-G.

Since $^3$H-DHT-G could be produced from the metabolism of $^3$H-Adiol, and since the $^3$H-DHT-G peak co-elutes from the HPLC with the Adiol-G peak, a correction had to be made for those counts in the Adiol-G peak which were due to $^3$H-DHT-G. To make this correction, the Adiol-G peak from control experiments was collected, hydrolyzed and the resulting products separated by TLC (General Methods, Section C, pg 101). From this study it was found that $1.4 \pm 0.3\%$ of the counts present in the Adiol-G peak were due to DHT-G; these counts were subtracted from the appropriate totals.

**METHOD 2**: The Adiol-G peak was collected and the counts attributable to Adiol 3-G were determined in the binding assay as described in General Methods, Section B, page 99.
Statistics: All results are expressed as the mean ± SD, unless specified otherwise.

RESULTS

Isomer production: The relative percentages of the two Adiol-G isomers formed from tritiated Adiol were determined in both the presence and absence of 5 μM unlabeled Adiol. 5 μM unlabeled Adiol was used to saturate competing enzymatic pathways.

The HPLC elution patterns of the Adiol 3-G tracer, Adiol 17-G tracer and the Adiol-G formed from 3H-Adiol in the glucuronyl transferase assay are shown in Figure 6. From these patterns it was determined that 65.9 ± 0.4% of the total counts from the glucuronyl transferase assay appeared between 10.75-11.75 minutes. Based on the equations described above, it was determined that 99.8 ± 1% of the Adiol-G formed was Adiol 17-G (See Appendix A for example of calculations).

The results from the binding assay are shown in Table 7. In either the presence or absence of 5 μM unlabeled Adiol, the binding of 3H-Adiol-G formed from 3H-Adiol in the glucuronyl transferase assay was no greater than the binding of the Adiol 17-G tracer, suggesting that little or none of the Adiol-G formed by the prostate tissue was Adiol 3-G.
FIGURE 6

HPLC ELUTION PROFILE OF RAT PROSTATE GLUCURONYL TRANSFERASE $^3$H-PRODUCTS: COMPARISON WITH $^3$H-ANDROSTANEDIOL 3-GLUCURONIDE AND $^3$H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES

THE ANDROSTANEDIOL 3-GLUCURONIDE ELUTION PROFILE IS SHOWN IN THE BOTTOM PANEL. THE ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILE IS SHOWN IN THE MIDDLE PANEL. THE ELUTION PROFILE OF THE CONJUGATES PRODUCED FROM THE METABOLISM OF ANDROSTANEDIOL BY RAT PROSTATE GLUCURONYL TRANSFERASE IS SHOWN IN THE TOP PANEL. THE DATA ARE PRESENTED AS PERCENT OF TOTAL ELUTING COUNTS. THE SHADED AREA DEFINES THE FRACTIONS ANALYZED FOR ANDROSTANEDIOL GLUCURONIDE ISOMER LEVELS.
FIGURE 6
TABLE 7

Percentage of $^3$H-Adiol-G present as $^3$H-Adiol 3-G following incubation of rat prostate glucuronyl transferase with Adiol. A binding assay using purified Adiol 3-G antibody was used to determine counts in the HPLC Adiol-G peak present as Adiol 3-G. Counts attributable to DHT-G have been subtracted from total Adiol-G counts. In control experiments, Adiol 3-G and Adiol 17-G tracers were incubated in the glucuronyl transferase assay with 0 or 5 $\mu$M unlabeled Adiol. The tracers were then re-isolated by HPLC and the percent binding of each to the purified Adiol 3-G antibody was determined. These values were compared to the products of similar incubations using tritiated Adiol as substrate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Percent of Counts Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. of unlabeled Adiol ($\mu$M)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Adiol 3-G tracer</td>
<td>$36.8 \pm 1.0$</td>
</tr>
<tr>
<td>Adiol 17-G tracer</td>
<td>$13.0 \pm 0.2$</td>
</tr>
<tr>
<td>Gluc-Trans Products</td>
<td>$11.5 \pm 0.6$</td>
</tr>
</tbody>
</table>
Substrate Specificity: After adjusting for recovery (60 ± 4%, as determined in three control incubations by adding Adiol 17-G tracer at the end of the incubations), the mean percent conversion of Adiol to Adiol-G in the absence of unlabeled Adiol was 3.5% / 10^6 cells / 90 minutes. Under similar conditions, conversion of DHT to DHT-G was only 0.22% and that of androsterone to Andros-G was only 0.24%.

SUMMARY

Rat prostate tissue contains a glucuronyl transferase enzyme capable of converting Adiol to Adiol-G. This enzyme is specific for the 17-carbon and shows substrate specificity towards Adiol.

These data suggest that DHT metabolism by peripheral tissues occurs by conversion of DHT to Adiol, followed by selective glucuronidation of the resulting Adiol at the 17-carbon position.
IV.

EXPERIMENT 2 PART B

PRODUCTION OF ANDROSTANEDIOL GLUCURONIDE
BY GLUCURONYL TRANSFERASE
PREPARED FROM HOMOGENIZED RAT LIVER
INTRODUCTION

Circulating levels of Adiol-G represent the average secretion of this metabolite by various tissues since many different tissues are capable of producing androgen glucuronides (see literature review). In Experiment 2 Part A, Adiol-G production by an androgen dependent extra-splanchnic tissue (prostate) was examined. In this experiment, the production of Adiol-G by rat liver tissue is examined. In addition, the levels of total Adiol-G as well as the levels of Adiol 3-G were determined in rat serum.

METHODS

Tissue was prepared, incubations were performed and samples processed as described in General Methods, Section C, page 101. The tritiated substrates used in the incubations were the same as those used in the rat prostate study (Experiment 2 Part A). Liver tissue and serum samples were obtained from 55-60 day old male Sprague-Dawley rats (Canadian Hybrid Farms, Halls Harbour, Nova Scotia). All enzyme assays were done in duplicate unless otherwise stated. All experimental comparisons were performed on the same day using the same enzyme preparation. The enzyme reaction was stopped and unconjugated steroids removed by extracting twice with 7 mL ether per extraction. The ether phase was dried under a stream of nitrogen and the
unconjugated steroids separated by TLC (General Methods, Section C, pg 101). The aqueous phase, containing the steroid conjugates, was processed as described for Adiol 3-G (General Methods, Section A-i, pg 75).

SAMPLE ANALYSIS: The production of Adiol-G isomers from Adiol was analyzed using the same two procedures described for rat prostate: 1) collecting 15 second fractions from the HPLC and analyzing the radioactive elution profile of the samples compared to $^3$H-Adiol 3-G and $^3$H-Adiol 17-G tracers and 2) by using the Adiol 3-G binding assay to determine the percentage of counts in the Adiol-G peak present as Adiol 3-G.

Method #1: The HPLC elution profiles for both Adiol 3-G and Adiol 17-G tracers were determined at the time the samples were being processed. At that time, 19% of Adiol 3-G eluted between 11.25 and 12 min, while 45% of Adiol 17-G eluted during the same time interval. The percentages of the two Adiol-G isomers present in the samples were determined by solving the following equations: 1) $X + Y = \text{total counts in Adiol-G fraction}$; 2) $0.19X + 0.45Y = \text{counts present in the 11.25 to 12 min interval}$, where $X = \text{counts attributable to Adiol 3-G}$ and $Y = \text{counts attributable to Adiol 17-G}$.

The percentage of counts present in the Adiol-G peak which were due to $^3$H-DHT-G was determined as described in
the rat prostate study. This value was less than 2% and was used to correct the counts present as Adiol-G.

Method #2: This analysis was done as described in Experiment 2, Part A, page 118.

Rat Serum Sample Analysis: Total Adiol-G and Adiol 3-G levels in rat serum were determined as described in General Methods, Sections A-i and -iii, pages 75 and 85. The volume of serum required for each determination was 2 mL.

Statistics: All results are expressed as the mean ± SD unless otherwise noted.

RESULTS

Adiol-G Isomer Production from Adiol: The relative percentages of the two Adiol-G isomers formed from tritiated Adiol were determined in the presence of 5 μM unlabeled Adiol. Under these assay conditions, there was little interconversion among the unconjugated steroids. When the glucuronide tracers were incubated in the enzyme assay (in the absence of unlabeled steroids), no interconversion among the glucuronides took place and there was no breakdown of glucuronide tracer to unconjugated steroid.
The HPLC elution patterns of the Adiol 3-G tracer, Adiol 17- G tracer and the Adiol-G formed from $^3$H-Adiol in the glucuronyl transferase assay are shown in Figure 7. From these patterns it was determined that $32 \pm 1\%$ of total counts from the glucuronyl transferase assay appeared between minutes 11.25 and 12. From the equations in the methods section, $61 \pm 5\%$ of the Adiol-G formed was Adiol 3-G.

Using the binding assay prepared from the IgG fraction of the Adiol 3-G antibody, the relative percentage of the two Adiol-G isomers formed from tritiated Adiol was determined in both the presence and absence of 5 µM unlabeled Adiol. In the absence of unlabeled Adiol, $61 \pm 2\%$ of the Adiol-G formed was Adiol 3-G. In the presence of 5 µM Adiol, $57 \pm 2\%$ of the Adiol-G formed was Adiol 3-G.

**Substrate Specificity:** After adjusting for recovery, the production rate (pmoles/mg liver tissue/min - determined by using the percent conversion of tracer applied to available unlabeled Adiol in the incubation) of Adiol 3-G from Adiol was $101 \pm 3$, while the production rate of Adiol 17-G from Adiol was $71 \pm 2$. The production rate of Andros-G from androsterone was $181 \pm 11$ and DHT-G from DHT was $6.9 \pm 0.4$. 
FIGURE 7

HPLC ELUTION PROFILE OF RAT HEPATIC GLUCURONYL TRANSFERASE $^3$H-PRODUCTS: COMPARISON WITH $^3$H-ANDROSTANEDIOL 3-GLUCURONIDE AND $^3$H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES

The androstanediol 3-glucuronide elution profile is shown in the bottom panel. The androstanediol 17-glucuronide elution profile is shown in the middle panel. The elution profile of the conjugates produced from the metabolism of androstanediol by rat hepatic glucuronyl transferase is shown in the top panel. The data are presented as percent of total eluting counts. The shaded area defines the fractions analyzed for androstanediol glucuronide isomer levels.
FIGURE 7
Serum Levels of Adiol-G Isomers: For each determination, sera from three rats were pooled to provide sufficient serum for extraction. Total Adiol-G levels (n=4) were 690 ± 60 pmol/L (Table 8). Adiol 3-G levels in the same samples were 470 ± 100 pmol/L. The percent Adiol-G present as Adiol 3-G was 69 ± 16 (mean ± SEM).

SUMMARY

Adiol was metabolized to both Adiol 3-G and Adiol 17-G by rat hepatic glucuronyl transferase. This suggests that both a 3-carbon glucuronyl transferase and a 17-carbon glucuronyl transferase are present in this tissue, in contrast to rat prostate tissue which had only a 17-carbon glucuronyl transferase. In addition, the hepatic 3-carbon glucuronyl transferase accepted both Adiol and androsterone as substrate, suggesting that it is not specific for Adiol. However, DHT was only poorly metabolized to DHT-G, suggesting that the 17-carbon glucuronyl transferase may be specific for Adiol. This is similar to what was found in rat prostate. Therefore rat liver appears to have a non-specific 3-carbon glucuronyl transferase which rat prostate does not have.

Since serum levels of Adiol-G isomers parallel the pattern of isomer production by the liver, it is likely that the liver is the major source of Adiol-G in the rat.
TABLE 8
Levels of Adiol 3-G and Total Adiol-G in Rat Serum as determined by RIA.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Adiol 3-G (pmol/L)</th>
<th>Total Adiol-G (pmol/L)</th>
<th>% of Total Adiol-G present as Adiol 3-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>337</td>
<td>692</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>586</td>
<td>692</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>469</td>
<td>756</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>469</td>
<td>603</td>
<td>78</td>
</tr>
<tr>
<td>X</td>
<td>465</td>
<td>686</td>
<td>69</td>
</tr>
<tr>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>S.D.</td>
<td>102</td>
<td>63</td>
<td>16</td>
</tr>
</tbody>
</table>
IV.

EXPERIMENT 2  PART C

PRODUCTION OF ANDROSTANEDIOL GLUCURONIDE
BY GLUCURONYL TRANSFERASE
PREPARED FROM HOMOGENIZED HUMAN LIVER
INTRODUCTION

The ratio of Adiol 3-G / Adiol 17-G produced from the metabolism of Adiol by rat hepatic glucuronyl transferase was the same as that present in the general circulation of the rat. In this experiment, Adiol metabolism by human liver was examined to determine if, as in the rat, the pattern of Adiol-G isomer production by hepatic glucuronyl transferase is the same as the ratio present in the general circulation.

METHODS

Liver biopsies were performed on patients undergoing cholecystectomy. All patients gave written informed consent using a protocol approved by the Halifax Infirmary Research Committee.

Tissue was prepared, incubations were performed and the samples processed as described in General Methods, Section C, page 101.

Sample Analysis: The Adiol-G produced from Adiol by human liver glucuronyl transferase was resolved into its Adiol 3-G and Adiol 17-G components by 1) analyzing the elution profile of both radioactive isomers eluting from the HPLC and 2) by collecting the Adiol-G fraction from the HPLC.
separation and directly assaying the fraction for both Adiol 3-G and Adiol 17-G in RIA's specific for each isomer.

Method #1: The HPLC was calibrated and a collection window was established for $^3$H-Adiol 3-G and $^3$H-Adiol 17-G by monitoring their elution profiles with a radioactive-flow detector (FLO- ONE\Beta Model IC, Radiomatic Instruments and Chemical Co., Inc, FL). This instrument is a scintillation counter which measures the radioactivity in the HPLC effluent as it elutes directly off of the column. This is done by mixing the effluent with a liquid scintillant and passing the mixture through a flow cell contained between the detectors photomultiplier tubes. For this experiment, the activity within the effluent stream was integrated over 6 second intervals, giving much greater peak resolution than the 15 second collection periods used in the previous experiments. This methodology was not used in the previous experiments since the equipment was not available at the time that those experiments were performed.

The collection window chosen for this experiment was 11.6 to 12.6 minutes. The equations derived from the HPLC elution profile of each isomer were: 1) $X + Y = \text{total counts in Adiol-G fraction}$; 2) $0.23X + 0.65y = \text{counts collected between 11.6 and 12.6 min}$, where $X$ is the counts in the collection window attributable to Adiol 3-G and $Y$ is the counts attributable to Adiol 17-G.
The data presented are from 4 separate incubations using a single liver preparation as the source of hepatic glucuronyl transferase.

**Method #2:** The levels of Adiol 3-G and Adiol 17-G produced from Adiol by the human liver glucuronyl transferase preparation was measured directly in the appropriate RIA's. Five replicates for each of six different liver samples were processed. The incubations were done using 5 μM unlabeled Adiol as substrate. To estimate procedural losses during sample preparation, 2000 cpm $^3$H-Adiol 17-G trace was added to each sample prior to extraction. The samples were handled as described above for serum (General Methods, Sections A-i and -ii, pgs 75 & 80). Following collection of the Adiol-G peak of the HPLC chromatogram, each sample was dried down, brought up in assay buffer and a fraction of the sample was taken for recovery determination. Based on the assumed percent conversion (4%) and the measured percent recovery, each sample was diluted sufficiently to bring it into the range of the standard curve. The assays were then carried out as described in General Methods, Sections A-i and ii, pages 75 and 80.

**Statistics**

All results are expressed as mean ± SD.
RESULTS

The HPLC elution profiles for the Adiol 3-G tracer, Adiol 17-G tracer and the Adiol-G formed from $^3$H-Adiol in the glucuronyl transferase assay are shown in Figure 8. From the equations given in the methods section above, it was determined that $87.6 \pm 4.1\%$ of the $^3$H-Adiol-G produced was Adiol 17-G and $12.5 \pm 4.1\%$ was $^3$H-Adiol 3-G.

The results from direct assay of Adiol 3-G and total Adiol-G in three of the incubations are shown in Figure 9. The percent of total Adiol-G present as Adiol 3-G from all 6 different liver samples was $6.4 \pm 4.6\%$.

SUMMARY

Human liver glucuronidates Adiol almost exclusively at the 17-carbon position. Since Adiol 17-G is the major isomeric form of Adiol-G found in serum (Experiment 1 Parts A and B, pgs 104 & 111) the hepatic compartment could contribute significantly to the overall production of Adiol-G in man.
FIGURE 8

HPLC ELUTION PROFILE OF HUMAN HEPATIC GLUCURONYL TRANSFERASE $^3$H-PRODUCTS: COMPARISON WITH $^3$H-ANDROSTANEDIOL 3-GLUCURONIDE AND $^3$H-ANDROSTANEDIOL 17-GLUCURONIDE ELUTION PROFILES

The androstanediol 3-glucuronide elution profile is shown in the bottom panel. The androstanediol 17-glucuronide elution profile is shown in the middle panel. The elution profile of the conjugates produced from the metabolism of androstanediol by human hepatic glucuronyl transferase is shown in the top panel. The data are presented as percent of total eluting counts. The shaded area defines the fractions analyzed for androstanediol glucuronide isomer levels.
FIGURE 9
ANDROSTANEDIOL-GLUCURONIDE ISOMER PRODUCTION FROM
ANDROSTANEDIOL BY HUMAN HEPATIC GLUCURONYL TRANSFERASE AS
DETERMINED BY RADIOIMMUNOASSAY

DATA FROM THREE REPRESENTATIVE INCUBATIONS ARE SHOWN.

ANDROSTANEDIOL 3-GLUCURONIDE: OPEN BARS

TOTAL ANDROSTANEDIOL GLUCURONIDE: HATCHED BARS
FIGURE 9
V.

EXPERIMENT 3

ANDROSTERONE GLUCURONIDE:
A MARKER OF ADRENAL HYPERANDROGENISM
INTRODUCTION

One of the subjects infused with $^3$H-DHT in Experiment 1 Part A produced a significant amount of a tritiated compound eluting off of the HPLC with a retention time identical to that of Andros-G, suggesting that Andros-G is a major metabolite of DHT. Since there is very little Adiol 3-G in the circulation, Andros-G likely represents the major circulating form of androgens glucuronidated at the 3-hydroxyl position. To further examine the production of 3-glucuronide conjugates and to determine the usefulness of such metabolites in the assessment of overall androgen metabolism in hyperandrogenic women, the levels of Andros-G were measured in normal women, overweight women (as controls for hirsute women who were often overweight), women with mild or severe idiopathic hirsutism and severely hirsute women with PCO. To determine whether the ovary or the adrenal is the primary source of Andros-G precursors, serum levels were measured in the following ways: in normal women during both the follicular and luteal phases of their cycles; in hirsute women prior to any treatment, then following selective ovarian suppression with leuprolide and again following ovarian suppression with leuprolide combined with adrenal suppression by dexamethasone; in hirsute women both before and after adrenal stimulation with ACTH.
METHODS

Subjects: The ages, degree of hirsutism, body mass index (BMI) and number of subjects for each group in which baseline hormone levels were measured are shown in Table 9. Subjects undergoing dynamic testing gave informed consent using protocols approved by the Halifax Infirmary Research Committee. For baseline measurements, all subjects were free of medications known to affect hormone levels for at least 3 months. Late-onset (attenuated) 21-hydroxylase deficiency was excluded with ACTH stimulation testing as described below. The normal women had regular menses, had a normal BMI, had no evidence of acne or hirsutism and were sampled in the early follicular phase of the menstrual cycle. Using a modification (Rittmaster 1988a) of the criteria of Ferriman and Gallwey (maximum score 36), mild hirsutism was defined as a hirsutism score of 8 to 15. Severe hirsutism was defined as a hirsutism score of 18 to 36. Women with idiopathic hirsutism (IH) had regular menstrual cycles and monthly moliminal symptoms, suggesting ovulation. Women with PCO had oligo- or amenorrhea, no moliminal symptoms, hirsutism and an elevated LH/FSH ratio (> 1.5; the LH/FSH ratio in 17 normal women sampled in the early follicular phase of the menstrual cycle was 0.8 ± 0.6, mean ± 2 SD). Non-hirsute obese women were evaluated prior
to gastric fundoplication for morbid obesity, had regular menstrual cycles, and were sampled in the follicular phase.

**Menstrual Cycle Study:** Serum samples from 11 normal women were drawn during the early follicular and mid-luteal phases of their menstrual cycles. Serum progesterone levels were used to verify the stage of the cycle from which the samples were drawn.

**Leuprolide/Dexamethasone Protocol:** Leuprolide is a superactive gonadotropin-releasing hormone (GnRH) agonist which down-regulates pituitary GnRH receptors. This has the result of lowering pituitary secretion of LH and FSH. In this experiment, 9 women with severe hirsutism (4 with PCO and 5 with IH) were given 20 μg/kg/day leuprolide as a daily subcutaneous injection for 5–9 months. The dose used in this study is sufficient to maximally suppress ovarian testosterone secretion (Rittmaster, 1988a). Dexamethasone, 0.5 mg/day, was then added as a single dose each evening for 4 weeks. Andros-G was measured before and after leuprolide administration, and after combined leuprolide/dexamethasone.

**ACTH Stimulation Test:** Nine normal women, 20 women with IH, and 17 hirsute women with PCO received 250 μg ACTH (Cortrosyn; Organon, West Hill, Ontario) intravenously between 1000 and 1400 hours. The IH and PCO groups included
women with mild to severe hirsutism. Andros-G was measured in duplicate immediately before and 60 minutes after the ACTH bolus. To exclude 21-hydroxylase deficiency, 17-hydroxyprogesterone was measured at the same time points.

**Sample Analysis:** Serum testosterone was measured using kits obtained from Immuchem Corp., Carson, CA, and serum DHEAS using kits obtained from Diagnostic Systems Laboratories, Webster, TX. The interassay coefficients of variation were 15% for testosterone and 9% for DHEAS.

Andros-G and Adiol-G levels were determined as described in General Methods, Sections A-iii and -iv, pages 85 and 92.

**Statistics:** Results are expressed as mean ± SD, unless otherwise noted. For baseline hormone levels, analysis of variance followed by Duncan's multiple range test (Steel and Torrie, 1960) was used to determine significant differences between groups (P<0.05). Differences between Andros-G levels measured during the two phases of the menstrual cycle as well as the results of stimulation and suppression testing were compared with the paired Student's t-test. Correlation between steroid levels was done by the least-squares method.
RESULTS

Baseline Steroid Levels:

Serum DHEAS levels (\(\mu\)mol/L) were 8.3 ± 3 in normal women and were not significantly different in the three groups of hirsute women (mild IH, 8.8 ± 3.4; severe IH, 8.6 ± 3.7; PCO, 7.9 ± 2.5). Serum DHEAS was 5.1 ± 2.2 in obese women (P<0.05, compared to normal women).

Serum testosterone levels (nmol/L) were 1.3 ± 0.6 in normal women and were not significantly different in obese women (1.2 ± 0.5) or in women with mild IH (2.0 ± 0.60). Serum testosterone levels in the severe IH group were significantly higher than the normal, obese and mild IH groups (2.2 ± 0.8; P<0.05 compared to normal women). The PCO group had testosterone levels which were greater than all of the other groups (3.5 ± 1.2; P<0.01 compared to normal women).

Serum Adiol-G levels (nmol/L) were 7.6 ± 2 in normal women (Figure 10, Panel I) and were not significantly different in obese women (7.8 ± 2.9). Serum Adiol-G levels were higher in mild IH (11.3 ± 6.1; P<0.05), in severe IH (12.4 ± 5.2; P<0.05) and in PCO (19.2 ± 6.7; P0.01), compared to normal women. The Adiol-G levels in PCO were significantly higher than in all other groups (P<0.01).
**TABLE 9**
Clinical Characteristics of Subject Groups (mean ± S.D.).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Subjects</th>
<th>Age (Years)</th>
<th>BMI (Kg/m²)</th>
<th>Hirsutism Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Women</td>
<td>15</td>
<td>26 ± 5</td>
<td>22 ± 3</td>
<td>&lt; 7</td>
</tr>
<tr>
<td>Obese Women</td>
<td>9</td>
<td>39 ± 9</td>
<td>45 ± 4</td>
<td>&lt; 7</td>
</tr>
<tr>
<td>Mild IH</td>
<td>13</td>
<td>28 ± 7</td>
<td>26 ± 5</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Severe IH</td>
<td>17</td>
<td>29 ± 8</td>
<td>29 ± 5</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>PCO</td>
<td>20</td>
<td>29 ± 6</td>
<td>31 ± 9</td>
<td>20 ± 9</td>
</tr>
</tbody>
</table>

IH - idiopathic hirsutism
PCO - polycystic ovarian disease
FIGURE 10

BASELINE ANDROGEN-GLUCURONIDE LEVELS IN NORMAL, OBESE AND HIRSUTE WOMEN

ANDROSTANEDIOL-GLUCURONIDE LEVELS ARE PRESENTED IN PANEL I.
ANDROSTERONE-GLUCURONIDE LEVELS ARE PRESENTED IN PANEL II.
A: SIGNIFICANTLY DIFFERENT (P<0.05) FROM NORMAL WOMEN (NW)
B: SIGNIFICANTLY DIFFERENT FROM OBESE WOMEN (OW)
C: SIGNIFICANTLY DIFFERENT FROM WOMEN WITH MILD IDIOPATHIC HIRSUTISM (MIH)
D: SIGNIFICANTLY DIFFERENT FROM WOMEN WITH SEVERE IDIOPATHIC HIRSUTISM (SIH)
WOMEN WITH POLYCYSTIC OVARIAN SYNDROME ARE IDENTIFIED WITH THE LABEL PCO.
Serum Andros-G levels (nmol/L) were 110 ± 26 in normal women (Figure 10, Panel II) and were significantly lower in obese women (64 ± 19; P<0.01). The levels were higher (P<0.025) in all groups of hirsute women compared to normal women: 185 ± 91 in mild hirsutism, 173 ± 97 in severe hirsutism and 178 ± 102 in PCO. Serum Andros-G levels were not significantly different when the groups of hirsute women were compared with each other.

When results in all groups of women were compared, serum Adiol-G and Andros-G levels were correlated with each other (r=0.62). There was a weaker correlation between serum testosterone levels and the glucuronides (r=0.49 for both Adiol-G and Andros-G) and there was minimal correlation between serum DHEAS and any of the other steroids measured (r=0.31 for testosterone, 0.11 for Adiol-G and 0.31 for Andros-G)

Changes in Andros-G Production During the Menstrual Cycle:
Andros-G levels during the follicular phase of the menstrual cycle were 118 ± 26 nmol/L. Luteal levels of Andros-G were 121 ± 39 nmol/L. These levels were not significantly different (P=0.676).
Changes in Andros-G Production Following Ovarian or Ovarian and Adrenal Suppression: Changes in serum Andros-G levels resulting from either ovarian suppression or ovarian suppression combined with adrenal suppression are shown in Figure 11.

In the nine women included in this study, baseline serum Andros-G levels were $171 \pm 15$ nmol/L (mean $\pm$ SEM). After 5-9 months of leuprolide alone, Andros-G levels were not significantly different ($153 \pm 18$ nmol/L; $P=0.11$). When dexamethasone was added to leuprolide, Andros-G levels were suppressed by a mean of $90 \pm 3$ percent from baseline to $19 \pm 6$ nmol/L ($P<0.001$). Andros-G suppression did not differ between those women with IH and those with PCO. Serum DHEAS levels (µmol/L) in these nine women showed a similar pattern: $8.1 \pm 2.1$ baseline, $7.7 \pm 1.4$ after leuprolide alone, and $1.1 \pm 0.8$ after leuprolide and dexamethasone.

Although an interaction between these two treatments cannot be ruled out, the pattern of DHEAS suppression suggests that the decrease in androgen conjugate production measured in this experiment is due to dexamethasone induced suppression of adrenal androgen production.

Adrenal Stimulation with ACTH: During ACTH stimulation (Figure 12), Andros-G levels (mean $\pm$ SEM) did not increase significantly in normal women ($112 \pm 14$ to $126 \pm 19$ nmol/L;
FIGURE 11

SERUM ANDROSTERONE-GLUCURONIDE LEVELS FOLLOWING EITHER OVARIAN SUPPRESSION OR COMBINED OVARIAN–ADRENAL SUPPRESSION

DEX – DEXAMETHASONE

* – SIGNIFICANTLY DIFFERENT FROM BASELINE
SERUM ANDROS-G (nmol/L)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Baseline</th>
<th>Lupron</th>
<th>Lupron/Dex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value (nmol/L)</td>
<td>150</td>
<td>175</td>
<td>20</td>
</tr>
</tbody>
</table>

FIGURE 11
FIGURE 12

SERUM ANDROSTERONE-GLUCURONIDE LEVELS BEFORE AND 60 MINUTES AFTER INTRA-VENOUS ACTH ADMINISTRATION IN NORMAL AND HIRSUTE WOMEN

THE INCREASE (MEAN ± SEM) SEEN IN NORMAL WOMEN (13 ± 5.7%) DID NOT ACHIEVE STATISTICAL SIGNIFICANCE (P=0.11), WHILE THE INCREASES SEEN IN THE IH GROUP (39 ± 8.4%, P<0.001) AND THE PCO GROUP (34 ± 10%, P=0.002) WERE SIGNIFICANT.

CONTROL VALUES: OPEN BARS

ACTH STIMULATION VALUES: HATCHED BARS
FIGURE 12

SERUM ANDROS-G (nmol/L)

NORMAL  I.H.  P.C.O.
+ 10%; P=0.17), although the results did not achieve statistical significance.

SUMMARY

Andros-G is produced from precursors secreted primarily from the adrenal and this secretion is more robust in hyperandrogenic women than in normal women.

Adiol-G and Andros-G were both poor markers for degree of hirsutism since neither distinguished between mild and severe IH groups. However, a distinction could be made between the IH and PCO groups based on Adiol-G levels.
VI.

SUMMARY
Adiol 17-G is the major circulating form of Adiol-G in both men and women. The production of Adiol 17-G appears to result from the selective glucuronidation of Adiol at the 17-carbon position rather than from conversion of DHT-G. Therefore, the most likely pathway of DHT metabolism is the conversion of DHT to Adiol followed by selective glucuronidation of the resulting Adiol at the 17-carbon position.

In the rat, both the prostate and liver produce Adiol-G from Adiol. Adiol-G isomer production by the liver more closely resembles the pattern of these isomers in the general circulation than does prostatic production. This suggests that the hepatic compartment contributes a greater percentage of Adiol-G to the circulation than does the prostate. Human liver also produces Adiol-G from Adiol and the pattern of isomer production by human liver also parallels the pattern seen in the circulation. These data suggest that Adiol-G is not made exclusively by the peripheral compartment.

Glucuronidation of the 3-carbon hydroxyl of Adiol does not occur readily in the human. However, the 3-carbon hydroxyl group of androsterone is readily glucuronidated. Andros-G is present in serum at levels which exceed by as much as a factor of ten the levels of total Adiol-G.

Andros-G is produced almost exclusively from the metabolism of adrenal steroids. Therefore, elevations of
Andros-G in hirsute women would be useful in identifying the adrenal as the source of the elevated androgen production.
DISCUSSION
I.

Technical Considerations

Certain technical aspects of this work require additional comment before full interpretation of the presented data can be made.

A) Internal Consistency of Data

The levels of total Adiol G and Adiol 3-G reported for normal men in Experiment 1, Parts A and B are different. This difference is likely the result of radioimmunoassay changes over the time interval separating the two experiments. Several years separate the two experiments and the antibody shared by the two assays (as explained in General Methods) had to be realiquoted several times over this period. The thawing and refreezing of the neat antibody as well as inaccuracies in diluting the aliquoted serum probably account for the shifts in the standard curves. Assay pools have been used to monitor assay performance and changes in assay characteristics have occurred only at those times when new antibody preparations were introduced. For these reasons, comparisons between data obtained by one antibody preparation with data obtained by a different antibody preparation have not been made. The
results from each experiment have been obtained using only one set of assay conditions.

When comparisons were made between RIA's (i.e. between Total Adiol G and Adiol 3-G and/or Adiol 17-G) calibration studies were done to adjust each of the isomer assays against the Adiol standard used in the total Adiol-G assay. This was done by hydrolyzing the Adiol 3-G and Adiol 17-G standards and assaying the resulting unconjugated Adiol in the total Adiol-G assay. For these reasons, the data within each experiment are internally consistent regardless of those differences which may exist between experiments. It is important to note that Adiol 3-G remained as only a minor constituent of total Adiol G regardless of assay conditions under which the values were determined.

B) External Consistency of Data

i. Adiol-G

Published serum levels of (total) Adiol-G vary between sources. Lobo et al (1983) reported levels of 1.4 nmol/L for normal, 2.4 nmol/L for non-hirsute PCO women and 18.7 nmol/L for hirsute women. Brochu et al (1987) reported levels of 6.2 nmol/L in normal women and 15.0 nmol/L in hirsute women while Horton et al (1982) recorded levels of 1.4 nmol/L in normal and 20.7 nmol/L in hirsute women. Kirschner et al (1987) published levels of 4.1 nmol/L in
normal and 16.7 nmol/L in hirsute women while Samojlik et al (1984) reported levels of 1.2 nmol/L in normal women and 15.0 nmol/L in women with idiopathic hirsutism. The values presented in this paper are 7.6 nmol/L for normal women, 11-12 nmol/L for IH women and 19 nmol/L for women with PCO.

The values for our normal women appear to be higher than most of the other reported values. As was mentioned in the General Methods Section, the commercially available Adiol-G assay used routinely by many research groups (Nichols Institute, California) had an Adiol-G standard which was more potent than ours. Upon calibration of our assay with theirs, we found that samples processed in our lab and measured simultaneously in their assay and ours were not significantly different and read within their established normal range. This suggests that those differences which exist between the data presented here and those previously published by others is not due to some impurity cross-reacting in our assay. Differences in the potency of standards may however account for some of the differences seen between the various reporting groups.

Currently, we are the only laboratory with the capability of directly determining total sample loss in the Adiol-G assay. Other laboratories correct for only that sample loss which occurs following the hydrolysis step. This is done by adding unconjugated tritiated Adiol to the sample hydrolyzate. An assumption of 100% efficiency must
be made by these laboratories regarding the hydrolysis step and those manipulations which precede it. By using our tracer, I have found this assumption to be invalid. Hydrolysis efficiency is usually between 85 and 90% with loss due to other manipulations accounting for an additional 10 to 15%. Of greater significance however, is the variability in hydrolysis efficiency that is seen between samples. This variability may be related to the buffering capacity of the various serum samples. If the pH is allowed to drift upwards from pH 5, there is a corresponding drop in hydrolysis efficiency. An assumption of 100% efficiency disregards this source of variability. This results in increased variability between samples and a decrease in assay precision. The final values calculated would underestimate the actual levels.

ii. Androsterone Glucuronide

Androsterone glucuronide is known to be a major end product of androgen metabolism. Most studies have examined the production of Andros-G by determining the daily excretion of Andros-G in the urine. However, Brochu et al (1987) have established a serum Andros-G assay and have measured the levels of this conjugate in serum obtained from normal and hirsute women. They found that the normal group had Andros-G levels of 25 nmol/L while the hirsute group had
levels of 117 nmol/L. These values are lower than those reported in Experiment 3 of this paper. In Experiment 3, the normal group had Andros-G levels of 110 nmol/L while the hirsute group (IH and PCO combined) had levels which were approximately 180 nmol/L. Dr. R.K. Matteri, working in Dr. R. Lobo's laboratory, has recently confirmed our published levels for normal and PCO women (personal communication, to be published), suggesting that the values presented by Brochu et al are inaccurate and low rather than ours being high. The procedure for measurement of Andros-G which was used by Brochu requires hydrolysis of the Andros-G and assay of the liberated Andros in an Andros radioimmunoassay. They do not possess tritiated Andros-G and as such cannot determine procedural losses. As a result, their assay has the same limitations as those described above for the total Adiol-G assays which are commercially available. We have not compared standards to determine whether potency differences in standard are responsible for these differences.

Etiocholanolone glucuronide levels are very high in serum, being as much as 1.6 times the level of Andros-G in young men (Deslypere et al 1982). In our chromatography system, etiocholanolone glucuronide elutes from the HPLC near the Andros-G peak. To be sure that our assay system was not being contaminated with etiocholanolone glucuronide (since our antibody displays 40% cross-reactivity with this
androgen conjugate), the HPLC fraction containing the Andros-G peak was hydrolyzed and the resulting unconjugated androgen(s) was measured in a radioimmunoassay specific for androsterone (the antibody used showed no cross-reactivity with etiocholanolone). The amount of androsterone measured in this assay agreed with the amount of Andros-G measured directly in the Andros-G assay. This confirmed that the levels of Andros-G reported in Experiment 3 are accurate and not contaminated by etiocholanolone glucuronide.

C) Glucuronyl Transferase Assay System

Prior to use in the glucuronyl transferase assay, rat prostate tissue was dispersed and then sonicated while rat and human liver were homogenized. The differences in methodologies were the result of simplifications made to the procedure. Initially, the glucuronyl transferase assay was performed using the procedure of Green (1985). This procedure required that the tissue be dispersed and sonicated. Since this procedure is time consuming and costly, it was modified. Subsequent experiments used only homogenized tissue. Studies were undertaken which compared the enzyme kinetics of the glucuronyl transferase produced by these two different methodologies. There was no difference based on the method of preparation. For this reason, subsequent experiments (rat liver and human liver)
were carried out using homogenized tissue rather than dispersed/sonicated tissue.

Five μM cold Adiol was used in those incubations where Adiol G isomer production was being examined. This was done for two reasons: 1) to saturate any other enzymatic reactions competing for the tritiated Adiol substrate thereby minimizing loss of this substrate to other enzymatic reactions and 2) to ensure that substrate availability to the glucuronyl transferase enzyme reaction was not rate limiting. A decrease in the specific activity of the tracer resulted from this addition, but sufficient radioactivity was used such that the final specific activity was still high enough to accurately measure the percent conversion to products.
II.

Site of Adiol-G Production

Splanchnic vs Extrasplanchnic Compartments

Peripheral tissues such as prostate and skin can produce DHT in situ from readily available precursors. Such tissues are also capable of metabolizing DHT before releasing it into the general circulation. This creates a DHT pool separate from that in blood. As a result, measurement of circulating DHT levels do not adequately describe the degree of peripheral tissue stimulation generated by DHT. For many clinical conditions, such as benign prostatic hyperplasia in men and hirsutism in women, the ability to assess the level of peripheral stimulation by DHT would be of great benefit to the clinician. As such, there has been much interest in identifying a marker in serum which adequately defines the level of peripheral DHT metabolism and action.

Adiol-G is believed to be a metabolite of peripheral DHT metabolism. Hirsute women should then have elevated Adiol-G levels as a consequence of the elevated DHT metabolism within their skin. Horton et al (1982) measured both DHT and Adiol-G levels in women with idiopathic hirsutism and compared their levels with those measured in normal women. They found that both DHT and Adiol-G levels
were elevated in the women with idiopathic hirsutism, but that over one half of the DHT values were within the normal range. In contrast, only one of these 25 hirsute women had Adiol-G levels which fell within the normal range. Similarly, Kirschner et al (1987) showed that only one of 28 women with IH had Adiol-G levels which were within the normal range. Lobo et al (1983) measured DHT and Adiol-G levels in normal as well as hirsute and non-hirsute women with PCO. They found that serum DHT levels were similar between the three groups. Adiol-G levels were similar between the normal and non-hirsute PCO women while the hirsute PCO group had significantly higher levels. Again, only one subject in the hirsute-PCO group had Adiol-G levels overlapping with the normal range (there was greater overlap between the two PCO groups, however). Meikle and Odell (1986) measured Adiol-G in a group of hirsute women containing both IH and PCO subjects. They also found good discrimination between groups, with only one subject of 16 having an Adiol-G value within the normal range.

These data support the hypothesis that Adiol-G is a useful marker of peripheral DHT metabolism (particularly by skin). However, there are several studies which do not show such a clear distinction in Adiol-G levels when comparing normal and hirsute women. Brochu et al (1987) reported Adiol-G levels in normal women, women with IH and hyperandrogenic women (women with high levels of
dehydroepiandrosterone). There was no difference in Adiol-G levels between normal women and women with idiopathic hirsutism. While the Adiol-G levels in the hyperandrogenic group were elevated above those of the normal and IH groups, 50% of the subjects fell within the normal range. Paulson et al (1986) reported that approximately 30% of hirsute women had Adiol-G values which fell within the normal range. Habrioux et al (1978), also failed to show a clear distinction in Adiol-G levels between normal and hirsute women. In their paper, Adiol-G levels are presented as a mean ± S.D. so the overlap between normal and hirsute groups cannot be determined directly. However, the values given for the hirsute group were 17.1 nmol/L ± 8.9 nmol/L while the values for the normal group were 8.2 ± 6.0 nmol/L. Based on these values, it can be assumed that the two groups showed considerable overlap. Statistical comparisons between these two groups failed to show a difference, something the authors attributed to the dispersion of the results in the hirsute group, again suggesting that there was considerable overlap with the normal group.

These discrepancies suggest that the relationship between Adiol-G and peripheral DHT metabolism may be more complex than originally suggested.

To better understand the relationship between Adiol-G levels and peripheral DHT metabolism, it is necessary to identify what contribution, if any, the liver makes to
Adiol-G levels. Based upon the work of Morimoto et al (1981), it has been generally accepted that the liver does not contribute significantly to Adiol-G production. It is possible however that they failed to identify such production based upon their experimental design. They infused men with radioactive testosterone and androstanediol. Using blood samples collected simultaneously from catheters placed in the decending aorta and hepatic vein they found no difference in Adiol-G levels across the splanchnic compartment. This suggested that the liver was not secreting Adiol-G. However, there is evidence from other sources that excretion of androgens and their conjugates can occur through the bile. Steinetz et al (1985) canulated the bile ducts of adult male and female Sprague-Dawley rats. $^{14}$C-Androstenedione was then injected via the jugular vein and the bile was collected over a two hour period. The radioactive metabolites of androstenedione, including androgen conjugates, which were excreted in the bile over this relatively short time period were determined. Male rats excreted 50%, while female rats excreted 20% of the injected $^{14}$C-load.

While these data suggest that androgens and their conjugates are readily secreted into the bile, very little androgen conjugate is actually excreted in the feces. This suggests that virtually all androgen conjugate secreted into
the bile is reabsorbed from the gut and consequently enters the general circulation.

Should the human liver produce Adiol-G, and should that Adiol-G be secreted into the bile rather than the hepatic blood supply, then hepatic Adiol-G could only enter the blood following its reabsorption from the lumen of the gastro-intestinal tract. Since bile is stored in the gall bladder, reabsorption of Adiol-G could only occur following the emptying of the gall bladder. In the fasted state, bile is not released from the gall bladder. As such, Adiol-G would not enter the gut, would not be reabsorbed by the entero-hepatic circulation and would not appear in the hepatic vein blood. In the Morimoto paper, the subjects were undergoing cardiac catheterization, and as such would have been fasted. It is possible, therefore, that Morimoto et al failed to detect hepatic production of Adiol-G due to the fact that they were examining this process under conditions which did not support detection of Adiol-G in hepatic venous blood. Administration of cholycystokinen or collection of bile from such patients would address this matter.

Should Adiol-G be produced by the liver and enter the circulation via the bile, the relationship between Adiol-G levels and peripheral DHT metabolism would no longer be direct. The elevated levels of Adiol-G present in hyperandrogenic women would reflect the elevated levels of
substrate available for metabolism by both the peripheral and hepatic compartments. The occurrence of hirsutism would depend upon the production of DHT by skin and the effectiveness of that DHT in stimulating hair follicle growth.

This model of Adiol-G production, which includes a contribution by liver, would allow for a dissociation of Adiol-G levels and hair growth. Since the factors controlling Adiol-G production and hair growth would be controlled separately, a more complex interaction between these events would exist than if the Adiol-G was being produced primarily from the overstimulated follicles as is currently suggested. It would be possible to have normal Adiol-G levels in hirsute women if the elevated Adiol-G produced in the skin was balanced by a drop in production by the liver. It is also possible that the skin normally contributes only a small fraction to total serum Adiol-G levels. In such a case, elevated Adiol-G production by hirsute skin may be swamped by hepatic production or production from other sources, thereby masking the relationship between hair growth and DHT metabolism in the skin. The possibility that the liver contributes to Adiol-G production is therefore an important consideration for the interpretation of DHT metabolism. As such, we decided to determine whether liver is capable of producing Adiol-G. To examine this possibility, an hepatic glucuronyl transferase
enzyme assay was established. Both rat and human liver homogenates were incubated with unconjugated Adiol and the production of Adiol-G was measured.

Glucuronyl transferase prepared from rat liver readily metabolized Adiol to Adiol-G. Similarly, human liver also metabolized Adiol to Adiol-G. Such findings were not unexpected given that others have demonstrated the presence of glucuronyl transferases in the liver. Matsui and Nagai (1986) purified hepatic microsomal glucuronyl transferase from Wistar rats. They isolated one form of this enzyme which metabolized testosterone (a 17-carbon glucuronyl transferase) and a second form which metabolized androsterone (a 3-carbon glucuronyl transferase). These findings are similar to those shown in this paper. Adiol was glucuronidated by rat liver at both the 3-carbon and 17-carbon positions. The fact that the 17-carbon enzyme described in this paper showed substrate specificity for Adiol suggests that it may be different from the one reported by Matsui and Nagai which metabolized testosterone.

The data given above clearly show that the liver is capable of producing Adiol-G from Adiol. However, it is not clear from these data whether such production actually contributes to the circulating levels of Adiol-G. An indirect method of addressing this issue would be to compare the pattern of Adiol-G isomer production by hepatic glucuronyl transferase with the levels of each isomer in
If liver tissue produces only one of the two possible Adiol-G isomers, and serum measurements show that isomer to be present in only trace amounts, then hepatic contribution to serum Adiol-G levels would be inconsequential.

Experiment 2, Part B showed that $69 \pm 16\%$ of total Adiol-G measureable in rat serum by radioimmunoassay was present as Adiol 3-G. When rat liver glucuronyl transferase was incubated with Adiol, $61 \pm 5\%$ of the Adiol-G produced was present as the Adiol 3-G isomer. In contrast, when rat prostate (an example of a peripheral tissue capable of metabolizing Adiol to Adiol-G) was incubated with Adiol, virtually all of the Adiol-G produced was Adiol 17-G. These data suggest that hepatic Adiol-G production contributes a much greater percentage of total circulating Adiol-G in the rat than does the peripheral compartment as represented by the prostate.

Experiment 2, Part C compared Adiol-G isomer production by human liver with the isomer levels in human serum. Human liver produced $88 \pm 4\%$ Adiol 17-G when incubated with Adiol, while Adiol 17-G accounted for about $83\%$ (total Adiol-G minus Adiol 3-G) of serum Adiol-G. This similarity supports, but does not prove, the hypothesis that the liver makes an important contribution to serum Adiol-G levels in the human.
Conclusion: Until now, many investigators believed that Adiol-G levels primarily reflect the metabolism of DHT by peripheral tissues. An elevation in Adiol-G levels is thought to reflect increased DHT stimulation within these tissues. However, data presented in this report show that the liver is also capable of producing Adiol-G. In addition, analysis of Adiol-G isomer production suggests that hepatic contribution to circulating Adiol-G levels may be substantial. Such a contribution would render the above model inaccurate and may explain some of the inconsistencies present in the literature regarding the correlation between Adiol-G levels and hirsutism scores.
III.
Pathways of Metabolism

In order for a steroid to exert androgenic activity, it must bind to the androgen receptor. For binding to occur, the steroid must have an available hydroxyl group at the 17-carbon position. Glucuronidation of the 17-carbon hydroxyl will block the binding of the steroid to the androgen receptor, resulting in termination of the steroid's androgenic activity.

Since glucuronidation of DHT can only occur at the 17-carbon position, glucuronidation of DHT itself would terminate the DHT signal immediately. Signal termination would not be as rapid if the initial step in DHT metabolism is the reversible production of other unconjugated androgens since these products could be back converted to DHT with the resulting equilibrium extending the biologic half-life of DHT. To develop a thorough understanding of how DHT is eliminated from androgen sensitive tissues, it then becomes important to determine the metabolic step at which glucuronidation occurs.

Serum levels of the two Adiol-G isomers contain information which help to identify the step(s) at which glucuronidation occurs. If DHT is glucuronidated directly, the glucuronide will be present only at the 17-carbon
position. Conversion of this DHT-G to Adiol-G will result in the production of the Adiol 17-G isomer. Significant levels of Adiol 3-G would suggest that the DHT is first metabolized to Adiol with the resulting Adiol glucuronidated at either the 3-carbon or 17-carbon positions.

Experiment 1 determined that approximately 80-90% of circulating Adiol-G is conjugated at the 17-carbon position. These data suggest that significant glucuronidation of DHT may be occurring. Alternatively, the DHT may be metabolized to Adiol and the Adiol then glucuronidated selectively at the 17-carbon position. It is also possible that DHT is metabolized to Adiol which is then glucuronidated at both the 3-carbon and 17-carbon positions, but that the Adiol 3-G isomer so produced is further metabolized.

To determine which of these possible mechanisms is the major route for in vivo metabolism of DHT, the production of DHT-G from DHT was compared to the production of Adiol-G from Adiol. If the enzyme metabolized one substrate more readily than the other, the substrate most readily glucuronidated by this in vitro system would likely represent the substrate most likely glucuronidated in vivo.

Glucuronyl transferase prepared from rat prostate tissue (Experiment 2, Part A) displayed substrate specificity towards Adiol. Adiol was glucuronidated at a rate 15 times greater than that of DHT. In addition, the Adiol-G produced was almost exclusively Adiol 17-G. These
data suggest that rat prostate, an androgen dependent tissue, metabolizes DHT by converting the DHT to Adiol and then selectively glucuronidating the Adiol at the 17-carbon position.

Glucuronyl transferase prepared from rat liver tissue (Experiment 2, Part B) also displayed substrate specificity towards Adiol. This tissue glucuronidated Adiol at a rate which was approximately 25 times that of DHT. Unlike rat prostate however, rat liver produced both Adiol 3-G and Adiol 17-G (61 ± 5% as Adiol 3-G). These data suggest that rat liver metabolizes DHT to Adiol and then glucuronidates the Adiol either at the 3-carbon position or the 17-carbon position. Since the glucuronyl transferase prepared from rat liver also accepted androsterone as a substrate (conjugation occurring at the 3-carbon position), it is likely that there are two separate glucuronyl transferase enzymes present in this tissue. One enzyme is substrate specific for Adiol and also specific for the 17-carbon position (produces Adiol 17-G only). This enzyme is similar to the one assayed in rat prostate. In addition, there is a second enzyme which does not show the same degree of substrate specificity, but does show carbon position specificity towards the 3-carbon position (produces Adiol 3-G as well as Andros-G).

Glucuronyl transferase prepared from human liver (Experiment 2, Part C) behaved in a manner more consistent
with rat prostate than rat liver. This enzyme metabolized Adiol almost exclusively at the 17-carbon position. Preliminary data not reported in Experiment 2, Part C suggest that human liver glucuronyl transferase is also capable of glucuronidating both DHT and androsterone. These substrates are metabolized about equally, both proceeding at a rate significantly lower than that of Adiol.

Based upon these data, a model of DHT metabolism can be constructed. DHT is metabolized by 3-ketosteroid reductase, producing androstanediol. This androstanediol is then conjugated to glucuronic acid by the enzyme glucuronyl transferase. In rats, peripheral glucuronyl transferase produces primarily Adiol 17-G while the liver produces both Adiol 3-G and Adiol 17-G. In man, Adiol 17-G is the major product. The low serum levels of Adiol 3-G reflect the low production of this isomer, rather than its rapid and efficient metabolism.

Further experimentation is required to verify some aspects of this model.
IV. Androsterone Glucuronide as a Marker of Androgen Metabolism

Experiment 1 showed that Adiol 17-G is the major circulating form of Adiol-G in human serum. Experiment 2, Part C showed that Adiol 17-G is the major isomeric form of Adiol-G produced from the metabolism of Adiol by human liver glucuronyl transferase. These data suggest that circulating Adiol-G levels reflect selective glucuronidation of Adiol at the 17-carbon position. From this, it can be concluded that Adiol 3-G is not a major product of Adiol metabolism and that glucuronyl transferase does not readily glucuronidate the 3-carbon position of Adiol. However, Andros-G is an androgen conjugate having a glucuronide at the 3-carbon position, and is a major product of testosterone (Baulieu and Mauvais-Jarvis 1964: Robel et al 1966: Deslypere et al 1982) and androstenedione (Vande Wiele et al 1962) metabolism. Therefore, there would appear to be two metabolic pathways leading to androgen glucuronide formation. One pathway (17-hydroxy steroid pathway) results in the production of the 17-glucuronide isomer of Adiol-G while the other pathway (17-ketosteroid pathway) results in the production of the 3-glucuronide, Andros-G (see Figure 1, pg 11). As such, Andros-G levels could yield useful
information about the overall pattern of androgen metabolism in vivo.

In Experiment 3, the levels of Andros-G in normal and hirsute women were measured and compared to the levels of Adiol-G in these same women. Andros-G levels were much higher than were the corresponding Adiol-G levels. This suggests that a greater fraction of steroid precursors is converted to Andros-G than Adiol-G.

Since Andros-G is a product of androstenedione metabolism, Andros-G levels may reflect a direct metabolic pathway leading from Androstenedione to Andros-G via the intermediates androstane-dione and androsterone (Figure 1, pg 11). Such a pathway would not contribute to the biologically active androgen pool.

Andros-G is also a major product of testosterone metabolism. Since the conversion of testosterone to androstenedione is greater than the reverse reaction, increased activity of the enzyme mediating this metabolic step (17-ketosteroid reductase) would elevate Andros-G production at the expense of Adiol-G by shunting androgen to the 17-ketosteroid pathway.

Based upon the above discussion, the pathway leading to Andros-G production may represent a 'conduit' leading to waste. The bulk of 17-keto steroids present in serum, including that produced from the metabolism of testosterone to Adione, would travel directly through this conduit,
bypassing the production of DHT. They would then be excreted as Andros-G.

There would be a great physiological benefit to such a conduit. Under this scenario, there would be a large reservoir of available steroid circulating in a biologically inert form. If not required, this steroid would be metabolized through to a waste product (Andros-G) that is simply ‘dumped’, via the urine. If required however, a simple shift in the activity of one enzyme, 17-ketosteroid reductase, would convert a pool of androgen precursors into biologically active androgens which could then be made readily available for tissue use. These androgens would ultimately be metabolized to the waste product specific for that pathway, Adiol 17-G.

Serum Andros-G and Adiol-G levels would be related only in as much as they both reflect the magnitude of the precursor pool. An elevation of precursor levels will result in elevated Andros-G production directly. Adiol-G levels will rise in relation to that percentage of the pool which is either secreted as testosterone or is converted to 17-hydroxy steroids from androgen precursors. The development of hirsutism will then depend on how the skin responds to the elevated production of DHT in response to the elevated testosterone and androstenedione levels. Again, it is suggested that hirsutism is dissociated from the actual levels of these androgen conjugates and correlate
to them only in as much as they both indicate the same overall process, i.e. elevated androgen precursor levels.

It should be noted that the serum levels of Andros-G were lower in the obese control group (acting as a control for the high body mass indices of the PCO group) than in the normal group. However, the levels of dehydroepiandrosterone (DHEAS), considered to be a steroid of adrenal origin only, were also lower. The fact that both Andros-C and DHEAS were similarly decreased in this group suggests that they shared a common source, the adrenal, and that the adrenals in the obese group were producing less steroid than were the adrenals of the normal women. Why obesity should have such an effect is not clear and has not been addressed in this thesis.

While both the ovary and adrenal secrete androstenedione, we have presented data which suggest that Andros-G is produced mainly from adrenal steroid precursors with the ovary contributing only minimally to its formation. Andros-G levels in the serum of normal women were measured during the follicular phase of the menstrual cycle and compared with the levels measured during the luteal phase. While the production of other ovarian steroids varies considerably between these two phases of the menstrual cycle, no change was detected in Andros-G levels. In a separate experiment, suppression of ovarian steroidogenesis in women with idiopathic hirsutism and hirsute women with PCO failed to
significantly decrease Andros-G levels, even though the degree of inhibition was sufficient to maximally suppress ovarian testosterone production (Rittmaster 1988a).

To examine the role of adrenal steroidogenesis in the production of Andros-G in women with idiopathic hirsutism and hirsute women with PCO, adrenal androgen production was inhibited with dexamethasone. This treatment was in combination with ovarian suppression, but unlike ovarian suppression alone, combined ovarian-adrenal suppression resulted in a marked reduction of serum Andros-G levels.

In a second study, normal women, women with idiopathic hirsutism and women with PCO were each given a bolus injection of ACTH and the changes in serum Andros-G were determined over the first hour of the stimulation. The IH and PCO groups both showed a significant rise in Andros-G levels over this time interval (39 ± 8.4% for idiopathic hirsute, and 34 ± 10% for PCO). While the normal group showed a modest increase in Andros-G levels (13 ± 5.7%), this increase was not significant. This does not necessarily mean that the adrenal is not the primary source of Andros-G precursor in normal women. Rather, it may be that one hour is insufficient time for normal women to show increased Andros-G production in response to an ACTH challenge. The fact that the hirsute groups had a demonstrable response over this short time interval suggests
that there is an increased production of adrenal androgens in hirsute women.

Elevated adrenal steroidogenesis in response to an ACTH challenge need not result in elevated cortisol secretion (the major feedback controller of ACTH secretion). ACTH acts to preferentially increase the activity of the adrenal 17-hydroxylase enzyme of the zona reticularis and zona fasciculata (Jefcoate, 1986). This results in a shift from corticosterone production to 17α-hydroxy progesterone production. This 17α-hydroxy progesterone would normally be metabolized to either cortisol or androstenedione. The enzyme required to change 17α-hydroxy progesterone to androstenedione is 17-20 lyase. Therefore, an increase in the activity of adrenal 17-20 lyase would increase the production of androstenedione in response to a normal ACTH challenge. Such an increase in androstenedione production would then result in decreased cortisol production. Decreased cortisol levels would increase ACTH stimulation of the adrenal until cortisol production was returned to normal. Under these conditions, cortisol secretion would be normal, but androstenedione production would be increased and the subject would become hyperandrogenic.

From the above data it is clear that Andros-G arises primarily from the metabolism of adrenal steroids. The fact that Andros-G levels are elevated in hirsute women suggests that adrenal androgen production is elevated in these women.
In addition, the adrenal response to ACTH is more robust in hirsute women than in normal women, suggesting that a fundamental change in adrenal steroidogenesis has occurred in the hirsute groups. That fundamental change may be an increased activity of the 17-20 lyase enzyme.

It is important to note that neither Adiol-G nor Andros-G levels were ideal markers for the presence of hirsutism since significant overlap between the normal and hirsute groups was evident for both of these androgen conjugates. Such overlap argues against the model of androgen glucuronide formation by peripheral tissues only (as elaborated upon above) and calls into question the value of relating androgen glucuronide levels to degree of hirsutism.

V.

SUMMARY

Androstanediol glucuronide is a product of dihydrotestosterone metabolism. Currently it is thought to be produced within the peripheral compartment only. However, data have been presented which show that the liver is capable of producing androstanediol glucuronide from unconjugated androstanediol. As such, the argument has been put forward that androstanediol glucuronide levels are a useful marker of overall androgen metabolism. This does not necessarily mean that they correlate well with DHT
metabolism within skin or the degree of hair growth in response to that DHT metabolism.

The metabolic pathway of the DHT signal will determine how quickly the signal is terminated. Data have been presented which show that DHT is converted to Adiol and then the Adiol so produced is glucuronidated. This mechanism will result in a more flexible control over the expression of the DHT signal. Since the conversion of DHT to Adiol is reversible, the DHT signal can be turned off via conversion to Adiol, but then turned back on again by the back conversion of Adiol to DHT. Therefore, the balance between the glucuronidation of Adiol and the back conversion of Adiol to DHT will ultimately determine the rate at which the DHT signal is terminated.

Andros-G is a major androgen glucuronide in serum. Data have been presented which show that Andros-G is produced from the metabolism of adrenal rather than ovarian steroids. The adrenals of hirsute women have a more robust response to ACTH in terms of Andros-G production than do the adrenals of normal women. It is suggested that the adrenal enzyme 17-20 Lyase may be more active in hirsute than normal women.
ALGEBRAIC SOLUTION FOR DETERMINING PERCENTAGE OF ANDROSTANEDIOL-GLUCURONIDE PRESENT AS ANDROSTANEDIOL 3-GLUCURONIDE

EXAMPLE: PERCENT OF ADIOL-G PRESENT AS ADIOL 3-G IN SERUM OF A NORMAL MAN FOLLOWING INFUSION OF TRITIATED DIHYDROTESTOSTERONE AS DETERMINED BY THE BINDING ASSAY. BINDING ASSAY ANALYSIS OF GLUCURONYL TRANSFERASE PRODUCTS IS DONE IN THE SAME WAY.

STEP | EXAMPLE
--- | ---
1 | DETERMINE TOTAL COUNTS IN THE ADIOL-G PEAK OF HPLC (COUNT AN ALIQUOT OF THE PEAK) 664
2 | CORRECT TOTAL COUNTS (OBTAINED IN 1) BY SUBTRACTING BACKGROUND RADIATION LEVELS FROM MEASURED TOTAL COUNTS (BACKGROUND = 15 CPM) 649
3 | DETERMINE THE COUNTS PRESENT IN THE ADIOL-G PEAK ATTRIBUTABLE TO DHT-G (8% OF TOTAL COUNTS) 52
4 | CALCULATE COUNTS IN ADIOL-G PEAK PRESENT AS ADIOL-G (SUBTRACT DHT-G COUNTS FROM TOTAL COUNTS) 597
5 | DETERMINE THE COUNTS PRESENT IN THE ADIOL-G PEAK WHICH WOULD BE BOUND NON-SPECIFICALLY BY IgG (1.8% OF TOTAL COUNTS) 12
6 | DETERMINE TOTAL NUMBER OF ADIOL-G COUNTS AVAILABLE FOR PRECIPITATION WITH IgG (SUBTRACT NON-SPECIFICALLY BOUND COUNTS FROM ADIOL-G COUNTS) 585
7 | CONSTRUCT EQUATION # 1
ADIOL 3-G COUNTS (X) + ADIOL 17-G COUNTS (Y) = TOTAL COUNTS PRESENT AS ADIOL-G
X + Y = 585
8 | DETERMINE NUMBER OF COUNTS BOUND BY IgG 184
9 | SUBTRACT COUNTS BOUND NON-SPECIFICALLY BY IgG (12 CPM) 172
10 | SUBTRACT BACKGROUND RADIATION LEVELS (15 CPM) 157
CONSTRUCT EQUATION # 2

BINDING ASSAY CALIBRATIONS DETERMINED THAT IgG BOUND 80% OF ADIOL 3–G (X) AND 8.4% OF ADIOL 17–G (Y) COUNTS AVAILABLE

\[0.8 X + 0.084 Y = 157\]

SOLVE THE TWO EQUATIONS BY SUBSTITUTION

\[0.8 X + 0.084(585-X) = 157\]

\[X = 151\]

DETERMINE PERCENT OF TOTAL ADIOL–G PRESENT AS ADIOL 3–G

\[151 / 585 * 100 = 25.8\%\]

THEREFORE, 26% OF CIRCULATING ADIOL–G WAS PRESENT AS ADIOL 3–G FOLLOWING THE INFUSION OF TRITIATED DHT.
REFERENCES


Verhoeven, G., Heyns, W. and DeMoor, P. (1977). Interconversion between 17β-hydroxy-5α-androstan-3-one (5β-dihydrotestosterone) and 5α-androstane-3α,17β-diol: tissue specificity and role of the microsomal NAD:3α-hydroxysteroid oxidoreductase. J. Ster. Biochem. 8:731-733.


