The in vitro metabolism of a typical adrenocortical steroid (11-deoxycortisol)
THE IN VITRO METABOLISM OF A TYPICAL ADRENOCORTICAL STEROID

(11-Deoxycortisol)

by

Enrico Henry Forchielli

(B.S. in Pharmacy, Massachusetts College of Pharmacy, 1940;
A.B. Clark University, 1951;
A.M. Boston University, 1953)

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

1956
Approved by

First Reader Ralph I. Dorfman
Research Professor of Biochemistry

Second Reader [Signature]
Professor of Biochemistry
ACKNOWLEDGEMENTS

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CHAPTER I

STATEMENT OF THE PROBLEM
Since the isolation and characterization of the first steroid hormone nearly 25 years ago, a vast literature has accumulated regarding the chemical, physiological and clinical aspects of the steroid hormones. With the development of new techniques such as paper chromatography and infrared analyses, the biochemical approach has received great impetus in recent years. The first biochemical efforts were directed to the identification of tissue and urinary steroids. These studies indicated certain relationships between glandular steroids and their metabolic products but direct proof was lacking. With the availability of adequate quantities of pure compounds, it became possible to administer these substances to experimental animals and human subjects and to study their metabolites in urine. This made possible the establishment of the relationship between tissue and urinary steroids and indicated the types of metabolic transformations that occurred in peripheral tissues. However, the results from such experimentation represented the contributions of many different tissues and organ systems, making it impossible to assess the role of a particular tissue or organ system. The logical approach to more definitive experimentation was utilization of the \textit{in vitro} technique in which the steroid transformations by individual tissues could be studied under relatively controlled conditions. The physiological significance of the interpretation of such studies is open to question since \textit{in vitro} experimentation involves removal of tissues and organs.
from their normal environment. Furthermore, disruption of cellular organization upon homogenization of tissue is well known to result in the inactivation of some enzyme systems, activation of others and release of inhibitors. The task of reconstructing a meaningful picture from the fragmentary results obtained from an isolated tissue or tissue component is a difficult one. Nevertheless, under such conditions, the opportunity is offered to study the potential contribution of a particular tissue in the metabolism of a particular steroid hormone uninfluenced by the activity of other tissue.

The role of the liver, as being the most important organ for the catabolism of the steroid hormone, has been established. Liver perfusion, incubations with slices, homogenates and purified liver preparations have all been demonstrated to be capable of effecting extensive changes on the steroid nucleus.

In this investigation, rat liver was selected as the tissue to be employed in order:

1. To develop a step-wise scheme for the *in vitro* metabolism of a typical adrenocortical steroid, 11-deoxy-cortisol, by incubating with a rat liver homogenate preparation and isolating and characterizing the conversion products.

2. To attempt to localize in various homogenate fractions individual enzyme systems which carry out single metabolic transformations.

The conversion products were isolated and purified from suitable extracts by column adsorption and paper partition
chromatography and crystallization. Identifications were established by infrared spectroscopic analysis, ultraviolet spectrophotometry in methanol and in concentrated sulfuric acid, melting points, specific color reactions and mobility rates in paper partition chromatographic systems.
CHAPTER II

STEROID NOMENCLATURE
The steroids are a class of biologically important compounds possessing the perhydrocyclopentanophenanthrene nucleus (Fig. 1). This group of compounds includes cholesterol, ergosterol and bile acids as well as androgens, adrenocortical hormones, estrogens, progesterone and their metabolites. Four basic steroid nuclei are illustrated in Figure 1: androstane, etiocholane, pregnane, and allopregnan. The carbon atoms are numbered with arabic numerals and the respective rings will be designated from left to right by the letters A, B, C, and D.

The steroid nucleus may be considered to lie in the plane of the paper with the exception of the angular methyl groups at C-10 and C-13 which are oriented above the plane of the paper and are reference points for the spatial arrangement of the chemical groupings. All substituents on the nucleus will be considered as being either above or below the plane of the paper and by convention those groups which lie above the plane of the molecule are represented by a solid line and those lying below the plane of the molecule are indicated by a dotted line.

It may be seen in Figure 1 that the androstane nucleus differs from that of etiocholane only with respect to the hydrogen at C-5. In the former it projects behind the plane of the molecule (α) and lies on the opposite side to the angular methyl groups. This configuration is designated as trans or allo. In the latter (etiocholane) the hydrogen at
Fig. 1. The basic steroid nuclei
C-5 lies above the plane of the molecule (β) and on the same side as the angular methyl groups and is designated as the cis or normal series. The pregnane nucleus and the allopregnane nucleus differ from the C19 steroids in having an ethyl side chain attached to C-17, but the relationship with respect to the hydrogen at C-5 is the same; one being designated as the trans, or the allopregnane series, and the other belonging to the cis, normal or pregnane series.

A second important form of isomerism is encountered with hydroxyl group substituents. When the hydroxyl group projects above the plane of the molecule it is designated as β; below the plane of the molecule it is designated as α. Such epimerization commonly occurs at positions 3 and 20.

A side chain at C-17 is designated as β in all naturally occurring steroids of the adrenocortical or progesterone type.

In naming compounds, the presence of a double bond is indicated by the Greek letter Δ (delta) along with a superscript indicating the location of the double bond and the suffix "ene". Ketone groups are located by the suffix "one" preceded by a number designating its position on the molecule. For example, the true chemical name of 11-deoxycortisol is: 17α,21-dihydroxy-Δ4-pregnene-3,20-dione.

SPECIAL DEFINITIONS:

1. Metabolism will be used to designate any chemical change in a steroid brought about by tissues or tissue prep-
2. Metabolites will be used to designate the conversion products resulting from the action of tissues or tissue preparations on steroid substrates.

3. The prefix dihydro will be used to designate reduction of the $\Delta^4$ group (uptake of two hydrogen atoms) in ring A of the steroids.

4. The prefix tetrahydro will be used to designate the reduction of the $\Delta^4$-3-ketone moiety (uptake of four hydrogen atoms) in ring A of the steroids.

5. The common name 11-deoxycortisol has been adopted as a common name for $17\alpha,21$-dihydroxy-$\Delta^4$-pregnene-3,20-dione. This stems from assignment of the common name cortisol to $11\beta,17\alpha,21$-trihydroxy-$\Delta^4$-pregnene-3,20-dione (190).
CHAPTER III

LITERATURE SURVEY
HISTORICAL

Addison's classical description "On the Constitutional and Local Effects of Disease of the Suprarenal Capsules" published in 1855 (1), established a relationship between the syndrome now known as Addison's disease and destructive lesions in the adrenal glands. In 1856 Brown-Sequard (23) first demonstrated that ablation of the adrenal glands in animals resulted in death. Isolation of epinephrine in 1901 (197) negated the role of the adrenal medulla in life maintenance, since administration of the hormone to adrenalectomized animals neither hastened nor postponed death. Nor was epinephrine of value in alleviating adrenal insufficiency symptoms in Addison's disease. No effective extract was available until 1929, when Rogoff and Stewart (154) prepared an adrenocortical extract which prolonged the life of adrenalectomized dogs. Beneficial results were also obtained in the treatment of Addisonian patients with these extracts. A preparation of an active adrenocortical extract was also obtained by Swingle and Pfiffner (196). Hartman and Brownell were able to prepare active extracts that were free from epinephrine (76). At that time, Hartman considered the active principle of the adrenal extract to be a single substance to which he gave the name cortin. Chemical studies on Hartman's preparation revealed a complex mixture. The resolution of the mixture and characterization of the individual components was accomplished by three groups of workers: Kendall and Mason and Wintersteiner and Pfiffner in this country and
Reichstein and his co-workers in Switzerland.

To date twenty-eight compounds have been isolated from adrenal extracts and identified (Table I). Only seven of these compounds possess adrenocortical activity (Fig. 2). All seven corticoids have certain chemical and structural features in common:

1. Twenty-one carbon atoms and the typical steroid ring structure
2. A $\Delta^4$-3-ketone grouping
3. An $\alpha$-ketol side chain at carbon 17 which is $\beta$-oriented.

These active adrenocortical steroids have been shown to have varying degrees of both glycogenic and electrolyte effects. However, the type and degree of biological activity is intensified or modified by certain structural features. The C-11 and C-17 oxygen functions do not appear to be essential for electrolyte activity, since deoxycorticosterone (V) which possesses neither of these two oxygen functions exerts a powerful sodium retaining and potassium excreting effect. The C-11 oxygen function greatly intensifies the glycogenic effect which is further augmented by the presence of a hydroxyl group at C-17. The C-18 oxygen function (as the aldehyde) thus far appears to be unique for aldosterone (VII) and this latter compound has been found to be 25 times more active than deoxycorticosterone with respect to sodium retention and 30 times more active with respect to glycogenic activity. The biological
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<td>A</td>
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<td>3β,11β,17α,21-tetrahydroxy-allopregnande-20-one</td>
<td>V</td>
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<td>3α,11β,17α,21-tetrahydroxy-allopregnande-20-one</td>
<td>C</td>
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<td>3β,17α,21-trihydroxyallopregnande-11,20-dione</td>
<td>D</td>
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<td>11β,17α,20β,21-tetrahydroxy-Δ⁴-pregnene-3-one</td>
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<td>17α,20β,21-trihydroxy-Δ⁴-pregnene-3,11-dione</td>
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<td>11β,17α,21-trihydroxy-Δ⁴-pregnene-3,20-dione (cortisol)</td>
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<td>17α,21-dihydroxy-Δ⁴-pregnene-3,11,20-trione (cortisone)</td>
<td>Fα</td>
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<td>11β,21-dihydroxy-Δ⁴-pregnene-3,20-dione-18-al (aldosterone)</td>
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<td>3β,17α,20β,21-allopregnane-tetrol</td>
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<td>3β,17α,21-trihydroxyallopregnande-20-one</td>
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<td>$3\beta,11\beta,21$-trihydroxyallopregn-20-one</td>
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<td>$3\beta,21$-dihydroxyallopregnane-11,20-dione</td>
<td>N H</td>
</tr>
<tr>
<td>$20\beta,21$-dihydroxy-$\Delta^4$-pregnene-3,11-dione</td>
<td>T</td>
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<tr>
<td>$11\beta,21$-dihydroxy-$\Delta^4$-pregnene-3,20-dione (corticosterone)</td>
<td>H B</td>
</tr>
<tr>
<td>$21$-hydroxy-$\Delta^4$-pregnene-3,11,20-trione (11-dehydrocorticosterone)</td>
<td>A</td>
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<td>$\alpha,\beta$-unsaturated ketone (constitution unknown)</td>
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**C$_{21}O_3$ Group**

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<td>$3\beta,17\alpha,20\beta$-allopregnane-triol</td>
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<td>$3\beta,17\alpha,20\alpha$-allopregnane-triol</td>
<td>O</td>
</tr>
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<td>$3\beta,17\alpha$-dihydroxyallopregnane-20-one</td>
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<td>$21$-hydroxy-$\Delta^4$-pregnene-3,20-dione (17$\alpha$-hydroxyprogesterone)</td>
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<td>$21$-hydroxy-$\Delta^4$-pregnene-3,20-dione (11-deoxyoorticosterone; DOC)</td>
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**C$_{21}O_2$ Group**

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<td>$3\beta$-hydroxyallopregnane-20-one</td>
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<tr>
<td>$\Delta^4$-pregnene-3,20-dione (progesterone)</td>
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**C$_{19}$ Group**

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<tbody>
<tr>
<td>$3\beta,11\beta$-dihydroxyandrostane-17-one</td>
<td></td>
</tr>
<tr>
<td>$\Delta^4$-androstane-3,11,17-trione (adrenosterone)</td>
<td></td>
</tr>
</tbody>
</table>
Table I (continued)

$\Delta^4$-androstene-3,17-dione
(androstenedione)

11$\beta$-hydroxy-$\Delta^4$-androstene-3,17-dione

dehydroepiandrosterone

**C$_{18}$ Group**

Estrone
Fig. 2. The biologically active adrenocortical hormones
effects of these hormones can be further modified or enhanced by introduction of the $\Delta^1$-unsaturation and/or halogenation at position 9 of the molecule.

**BIOSYNTHESIS OF ADRENOCORTICAL STEROIDS**

With the host of compounds isolated and identified from adrenal gland extracts, the question was raised as to whether these compounds were representative of the true adrenal secretory product or were they artifacts concurred in the handling of the tissue prior to extraction. One of the greatest contributions towards elucidating the nature of the true adrenal secretory product was made by the group at the Worcester Foundation for Experimental Biology (91, 87). By perfecting the technique for the perfusion of an isolated adrenal gland it was possible for this group to analyze the effluent from the perfused gland for secretory products. By addition of adrenocorticotropic hormone (ACTH) to the perfusion medium augmented production of corticoids could be demonstrated (138), and the major products were found to be cortisol and corticosterone.

Other workers found that the intact gland was not necessary for the production of corticoids since adrenal slices (83, 19) incubated in the presence of ACTH could be shown to produce corticoids. Likewise, adrenal homogenates have been demonstrated to produce corticoids (119), but this type of preparation did not respond to ACTH.

Confirmation of the results of the *in vitro* studies
comes from in vivo experiments involving analysis of adrenal venous blood. Following the intravenous administration of ACTH to a dog, Nelson et al. (132) were able to isolate from adrenal venous blood cortisol and corticosterone. Their findings were confirmed by Zaffaroni and Burton (229) who also found cortisol and corticosterone in dog adrenal venous blood by paper chromatographic methods. Similar results were obtained by Romanoff and Pincus (156) from the analysis of human adrenal venous blood.

A number of studies have been directed toward ascertaining the precursors and origins of the adrenocortical steroids. A relatively high concentration of cholesterol was found to be present in the adrenal gland which was markedly depressed upon ACTH stimulation (117). This observation, in addition to the demonstrated conversion of acetate to cholesterol in the adrenal gland, has led to a study of these two substances as corticoid precursors. Hechter et al. (90) and Zaffaroni et al. (230) first demonstrated that both acetate and cholesterol can act as precursors for corticoids. Perfusion of C¹⁴-acetate and C¹⁴-cholesterol through the isolated cow adrenal gland resulted in the identification of radioactive cortisol and corticosterone. Similarly, incubation of C¹⁴-acetate with hog (75), beef (83) and rat adrenal (84) slices led to the finding of radioactive cortisol and corticosterone. Furthermore, Hechter et al. (90) observed that both corticoids had identical specific radioactivities. These results were
consistent with the idea that both corticoids arose from a common intermediate, which on the basis of the best evidence appeared to be progesterone.

Differences were noted in the rates of incorporation of acetate and cholesterol into corticoids in the presence of ACTH, cholesterol incorporation being more markedly influenced relative to acetate. It was suggested that the conversion of acetate to corticoids proceeded through an alternative pathway without the necessary involvement of cholesterol as an obligatory intermediate. More recent studies by Stone and Hechter (192) strengthen the view that acetate is converted through alternative pathways, only one of which involves cholesterol. These workers found that ACTH increased the incorporation of cholesterol into corticoids about 18-fold whereas ACTH increased the incorporation of $\text{C}^{14}$-acetate only about 1.4-fold. The effect of ACTH appeared to be concentrated at a very early step in the synthesis of corticoids from cholesterol most likely at a point involving splitting of the side chain between C-20 and C-22. The basis for this was that ACTH had no effect on the conversion of progesterone to corticoids nor did it influence any of the hydroxylation reactions leading to the corticoids.

Still to be desired was an understanding of the manner in which the steroid nucleus was synthesized from smaller molecules. To this end much effort has been expended in studying the biosynthesis of cholesterol from labeled pre-
cursors. Bloch's group (116, 222) have inferred from chemical degradation data of cholesterol, biosynthetically produced from doubly labeled acetate, that every carbon atom in the cholesterol molecule could conceivably be derived from both the carboxyl and methyl carbons of acetate.

A large number of small molecules such as ethanol (15, 46, 153), butyrate (153, 20), valerate (153, 225, 224), hexanoate (20), acetaldehyde (15, 21), acetone (27, 17, 144) and pyruvate (153, 20), have been tested as possible precursors in the biosynthesis of cholesterol. The results obtained suggested that only compounds closely related metabolically to acetate (ability to form "active acetate") were efficient precursors of cholesterol. On the other hand acetoacetate was found not to be degraded to acetate prior to incorporation into cholesterol (21, 45) which along with butyrate, isovalerate and leucine (153) was found to be more efficiently utilized for cholesterol synthesis than acetate even though the latter three compounds are known to be metabolized by way of two carbon units. It would appear that the common intermediate at this point might well be acetoacetate. In support of this, it has been demonstrated that the isopropyl moiety of isovalerate can be incorporated into acetoacetate (224) and in addition carbon dioxide fixation by the isopropyl group of isovalerate (223) and leucine (44, 43) to yield acetoacetate has been demonstrated. Wuerich et al. (222) have suggested the
possibility that condensation of acetate with acetoacetic acid could result in the formation of C₅ units related to isoprene. Condensation of several isoprene units could then result in the formation of the C₃₀ triterpene squalene which with proper folding and cross-linking and loss of three methyl groups would yield cholesterol. Both Langdon and Bloch (107) and Schwenk et al. (188) have demonstrated the transformation of squalene to cholesterol both in vivo and in vitro. In addition, Schwenk et al. have demonstrated that significant amounts of radioactive squalene are formed during cholesterol synthesis after perfusion of acetate-C¹⁴ through mammalian liver. More recent work has contributed a little more towards clarification of the intermediates between acetate, squalene and cholesterol. Rabinowitz et al. (145) have demonstrated that β-hydroxy, β-methyl glutarate, biosynthesized from acetate-C¹⁴ can be incorporated into squalene and cholesterol by a soluble rat liver preparation. In addition they have obtained indications that the incubation of β-hydroxy, β-methyl glutarate with farnesenic acid (a sesquiterpene acid) added as carrier, resulted in marked reduction of substrate incorporation into squalene. Furthermore labeled farnesenic acid was isolated from the same experiment. Bloch et al. (16) have demonstrated the incorporation into cholesterol of labeled dimethyl acrylic acid, β-hydroxy, β-methyl glutarate and β-hydroxyisovaleric acid fed to rats, the dimethyl acrylic acid being incorporated to the greater extent. Some information
is available as to possible precursors between squalene and cholesterol. Clayton and Bloch (40) have found that lanosterol-C\textsubscript{14} was synthesized from acetate-C\textsubscript{14} and in turn the lanosterol was converted to cholesterol in surviving liver tissue. Furthermore Schwenk et al. (187) have observed that zymosterol (lanosterol minus three methyl groups) when administered to rats was incorporated into cholesterol in good yield. In addition, from the Bloch group comes the finding that feeding of Δ\textsuperscript{7}-cholestenol and Δ\textsuperscript{7}-dehydrocholesterol to rats inhibited the synthesis of cholesterol from acetate (108). Figure 3 indicates the most likely distribution of acetate molecules in cholesterol (221).

Fig. 3. Origin of carbon atoms in cholesterol when biosynthesized from acetate. X = acetate carboxyl; O = acetate methyl.
Even though it was well established that adrenocortical steroids are derived from acetate and cholesterol, an understanding of the intermediate steps in the biosynthetic chain leading to active hormones still remains obscure. What is the nature of the hormonal precursors and what transformations do these compounds undergo in the production of cortical hormones? To obtain answers to the questions raised, known steroid compounds, which were plausible intermediates between cholesterol and cortisol, were presented to the adrenal gland using either perfusion of intact adrenals or incubations with slices, breis, homogenates or cell fractions and the transformation products isolated. From these investigations indications were obtained as to the types of reactions that the adrenal was capable of carrying out as well as the nature of the compounds upon which the various transformations could be effected. From the study of a large number of compounds the following types of reactions were observed which would lead to biologically active adrenocortical steroids:

1. Oxidation of the cholesterol side chain (170, 118).
2. Oxidation of $\Delta^5$-3$\beta$-ol to a $\Delta^4$-3-ketone (141, 174, 111, 127).
3. Hydroxylations at C-11 (78, 91), C-17 (78, 91, 141) and C-21 (91, 78, 79, 141).

In addition certain preliminary conclusions concerning the sequence of hydroxylations could be made. When a molecule such as progesterone was initially hydroxylated at
position 17, further hydroxylations at C-11 and C-21 could occur to yield cortisol. But initial hydroxylation at C-21 is apparently followed by preferential hydroxylation at C-11 to yield corticosterone. Hechter et al. (88) were not able to demonstrate C-17 hydroxylation of corticosterone. However, Dorfman et al. (55), Heard et al. (84) and Haines et al. (75) did observe conversion of deoxycorticosterone to cortisol. For a more detailed review of the subject, reference can be made to the excellent reviews of Hechter and Pincus (89) and Lieberman and Teich (115).

A tentative scheme is presented in Figure 4 for the corticosteroid biosynthesis compiled from evidence obtained from reactions of steroid intermediates with adrenal tissue. Cortisone (II) and 11-dehydrocorticosterone (III) have not been included in the figure since they are considered to be equilibrium products of cortisol and corticosterone respectively rather than being located in the main biosynthetic pathway.

Adrenal gland extracts have also been shown to possess progestational, estrogenic and androgenic activities. This has been substantiated by the isolation of representative compounds from each class. Beall and Reichstein (8) isolated progesterone and Beall (6) has shown the presence of estrone in adrenal gland extracts. The C19 androgens, Δ^4-androstene-3,17-dione, adrenosterone, and 3β,11β-dihydroxyandrostan-17-one, have been successfully isolated from similar extracts by Reichstein and Shoppee (150). Bloch et al. (13) were able to
Fig. 4. A tentative scheme for the biosynthesis of the adrenocortical hormones.

- **CHOLESTEROL**
  - Leads to **PREGNENOLONE**
  - Leads to **PROGESTERONE**
  - Leads to **DEOXYCORTICOSTERONE**
  - Leads to **CORTICOSTERONE**
  - Leads to **11-DEOXYCORTISOL**
  - Leads to **CORTISOL**

- **CH$_3$COO**

- **C$=O$**

- **CH$_3$**

- **HO**

- **CHO**

- **CH$_2$OH**

- **C$=O$**

- **OH**

- **OO**

- **CO**

- **=**

- **-**

- **X**
show the presence of $\Delta^4$-androstene-3,17-dione in a human fetal adrenal and furthermore Bloch et al. (14) demonstrated the ability of the perfused isolated beef adrenal gland to produce C19 androgens. Out of five possible androgens present in the perfusates, $\Delta^4$-androstene-3,17-dione, adrenosterone and 11$\beta$-hydroxy-$\Delta^4$-androstene-3,17-dione were identified. In vivo evidence for the production of androgens by adrenal tissue was presented by Pincus and Romanoff (140) who isolated and identified 11$\beta$-hydroxy-$\Delta^4$-androstene-3,17-dione from human adrenal venous blood.

STEROID BIOSYNTHESIS IN EXTRA-ADRENAL TISSUE

Testis:

Testosterone, the most potent naturally-occurring androgen, has been isolated from the testis of several mammalian species. Definitive proof of the biosynthetic role of the testis has been afforded by the work of Brady et al. (18) who demonstrated the incorporation of C$^{14}$-acetate into testosterone after incubation of the substrate with hog, rabbit and human testis tissue. This was confirmed by Savard et al. (178) by perfusing C$^{14}$-acetate through the human testis in vitro and isolating testosterone and $\Delta^4$-androstene-3,17-dione from extracts of the perfusates. In vivo, West et al. (217) have isolated testosterone and $\Delta^4$-androstene-3,17-dione from dog spermatic vein blood. The secretory activity of the testis apparently is not limited to elabora-
tion of androgens. Pregnenolone which bears a precursor relationship to progesterone has been isolated from testis tissue (167), and furthermore, Samuels et al. (174) have demonstrated the presence of the requisite enzyme system for oxidizing pregnenolone to progesterone in testicular tissue. Even the biosynthesis of estrogens has been indicated in testis as is evidenced by the isolation of estrone and estradiol-17β from stallion testis (7) and estradiol-17β from human testis (73).

Ovary:

Estrogens have been isolated and identified from ovarian tissue of several species. More recently direct evidence for the role of the ovary in elaborating these substances was derived from the work of Rabinovitz et al. (146). They demonstrated the biosynthesis of radioactive estrone and estradiol-17β from acetate-C\textsuperscript{14} incubated with ovarian tissue. Complimentary in vivo data is that of Rakoff and Cantarow (147) who observed estrogenic activity in dog ovarian blood.

Progesterone production has been associated with formation of the corpus luteum and in addition progesterone and one of its reduced metabolites, 3β-hydroxyallopregnan-20-one, has been isolated from sow (219) and whale ovary (143). In addition a 6β-hydroxylating enzyme system has been demonstrated in corpus luteum tissue (82), indicating the presence of 6β-hydroxylated steroids in this gland. No special physiological function has been attributed to such steroids.
Placenta:

The placenta elaborates progesterone and the estrogens, estradiol-17β, estrone and estriol. More directly, Solomon et al. (172) demonstrated the ability of the placenta to synthesize progesterone from cholesterol. These workers isolated both labeled pregnenolone and progesterone after the perfusion of cholesterol-C\textsuperscript{14} through the placenta in vitro. The production of estrone and estradiol from acetate-C\textsuperscript{14} by human term placenta has been recently reported by Levitz et al. (109). The presence of glucocorticoids in human placenta has been shown by Johnson and Haines (99), indicating the presence of 11 and 21-hydroxylating enzymes. Consistent with this is the finding of an increased excretion of urinary glucocorticoids in human pregnancy (214).

The continued excretion of 17-ketosteroids following adrenalectomy and gonadectomy suggests that other non-endocrine tissue may play some role in the elaboration of steroid hormones. Dorfman et al. (56) have observed that 1/3 of the preoperative amount of 17-ketosteroids was excreted in the absence of both the gonads and adrenals in the monkey.

CATABOLIC ASPECTS OF STEROID HORMONE METABOLISM

The steroid hormones are known to undergo a multiplicity of reactions in both mammalian and microbiological systems. These reactions involve practically every carbon on the steroid nucleus. Hydroxylation reactions on the steroid nucleus have been shown to occur at carbons 6, 7, 8, 11, 14, 15, 16, 17, 18, 19 and on the side chain at carbon 21.
Hydrogenation of unsaturated centers and even the formation of centers of unsaturation have been demonstrated in addition to reversible reduction of carbonyl groups and splitting of carbon to carbon links.

Since the research to be presented in this dissertation will involve many of the above mentioned reactions, a general review of the more pertinent ones will be discussed and for the sake of conciseness and brevity, the discussion will be limited to include only key references.

As the changes in the steroid molecule which result in loss or modification of activity are mediated by specific enzyme systems, the discussion to follow will be organized on the basis of a classification suggested by Dorfman (50). This classification groups the reactions in terms of enzyme systems in the following manner:

1) Δ-Hydrogenases; enzyme systems that reduce double bonds.

2) Hydrogenases and dehydrogenases; enzyme systems which reversibly reduce carbonyl groups to secondary alcohols.

3) Desmolases; enzyme systems that rupture carbon to carbon linkages.

4) Deoxylases; enzyme systems that reduce alcoholic groups to hydrocarbon residues.

5) Hydroxylases: enzyme systems that selectively introduce hydroxyl groups at specific carbon atoms in the steroid nucleus.
ACTION OF THE Δ-HYDROGENASES

Fig. 5. Reduction of the Nuclear Double Bond.

One of the major pathways for the in vivo catabolism of steroid hormones is in the formation of biologically inactive ring A reduced compounds. The reduction may proceed to either 5β (pregnane or etiocholane) or 5α (allopregnane or androstane) derivatives.

The C19 compounds, testosterone and Δ4-androstene-3,17-dione when administered to human subjects resulted in an increased excretion of reduced products of both the etiocholane (5β) and androstane (5α) type (51,34,48,182,54,72). Similar findings have been obtained after administration of testosterone to the chimpanzee (66), the monkey (53,95) and the guinea pig (52,30).

Ring A reduction of adrenocortical steroids after administration to human subjects resulted in the excretion of a preponderance of 5β (pregnane or etiocholane) reduced
metabolites. This has been demonstrated after the administration of cortisone (29, 27, 5, 151, 152, 47, 113), cortisol (152, 27, 5, 151, 152), 11-deoxycortisol (152, 11, 12, 9), deoxycorticosterone (152, 57), corticosterone (152) and dehydrocorticosterone (152, 121). Administration of progesterone to human subjects has also resulted in a preponderance of 5β reduced metabolites in urine (59, 213, 33, 130, 211). Similar results were obtained after administration of progesterone (85) and deoxycorticosterone (218, 92) to rabbits.

Dorfman has proposed (49) that, in the human, the side chain at C-17 and the C-11 oxygen function may have steric influence on the course of the reduction of the double bond at C-4(5). This is based on evidence that after the administration of C21 steroids, the reduction of the Δ4-3-ketone function in ring A results in the formation in vivo of a great preponderance of 5β derivatives. Following administration of C1903 compounds (e.g. adrenosterone), the 5α reduced metabolites exceeded the excretion of 5β reduced metabolites by a ratio of approximately 5:1, whereas with the C1902 compounds (testosterone, Δ4-androstene-3,17-dione), the ratio of 5α to 5β reduced metabolites was approximately 1:1. However, Engel et al. (63) administered large doses of corticosterone to a patient and found a concentration of 5α reduced products equal to those of the 5β stereoisomers. The finding appears to be unique and in exception with the experiences mentioned above in which the predominant reduction products of the C21 steroids have always been found to be in
the 5β series. The uniqueness of this report becomes more apparent since Richardson et al. (152) have studied the in vivo metabolism of corticosterone and 11-dehydrocorticosterone in humans and found that the 5β reduction products in urine exceeded by far the 5α reduction products.

In vitro techniques, utilizing either perfusion of intact organs or incubation with various tissue preparations, resulted in the isolation of reduced products mainly of the 5α (androstane or allopregnane) type. This has also been true for those ring A reduced compounds isolated from adrenal gland extracts (Table I). Notable exceptions have been reported. Incubation of progesterone with rabbit liver slices (96) afforded the pregnane derivative pregnane-3α,20α-diol, and in the case of testosterone incubated with chick liver slices, the metabolites isolated were of the etiocholane (5β) form (173). More recently Taylor (201) incubated progesterone with a rabbit liver homogenate and isolated and identified a total of five conversion products which included both 5α and 5β reduced products, the 5β stereoisomers being the preponderant products. In vitro experiments with rat liver tissue have resulted in the production of only 5α derivatives. Incubation of rat liver slices with deoxycorticosterone (184), rat liver homogenate with progesterone (38) and the perfusion of cortisone (37,200) and cortisol (36) through the intact rat liver has yielded only 5α reduction products. It was thought for some time that preferential reduction to the 5α or 5β products represented a species difference. But recently
Tomkins and Isselbacher (203) demonstrated the conversion of cortisone to the tetrahydropregnane (5β) derivative following the incubation of the former with a particulate free supernatant derived from a rat liver homogenate.

It would appear that more than one enzyme system is capable of catalyzing the reduction of the Δ⁴-3-ketone system. Samuels et al. (175) showed that the destruction of the α,β-unsaturation in ring A of testosterone (which was measured by loss of ultraviolet absorption at 240 μm due to the Δ⁴-3-ketone system) was accelerated by DPN and citrate and a need for oxygen was established. The destruction of the conjugated system in progesterone under anaerobic conditions was demonstrated by Wiswell and Samuels (220) and in this instance, neither a source of high energy phosphate nor DPN was required but the reaction was accelerated by the presence of citrate and other tricarboxylic acids. The suggestion was made that the latter promoted the metabolism of progesterone by forming complexes with metal ions, since cysteine and cyanide had a parallel effect. Taylor (201) confirmed the ring A reduction of progesterone in rabbit liver homogenate, but found that DPN and nicotinamide effected an increased rate of reduction of ring A in progesterone. Furthermore, Isselbacher and Tomkins (203) reported the presence in a rat liver homogenate preparation (cell free supernatant) of an enzyme system precipitable by 70 percent saturation with ammonium sulfate which catalyzed the reduction of cortisone
to the tetrahydropregnane derivative. The hydrogen donor was found to be TPNH, DPNH being inactive. The reduction could be coupled to the oxidation of d-isocitrate by TPN and isocitrate dehydrogenase or to the oxidation of glucose-6-phosphate dehydrogenase. The reaction proceeded faster in nitrogen than air. This TPNH-linked enzyme was also capable of destroying the ring A unsaturation of progesterone, testosterone, adrenosterone, cortisone and deoxycorticosterone.

That this phenomenon (reduction of the ring A double bond) is not an exclusive property of mammalian tissue is exemplified by the work of Murray and Peterson (131). When these workers incubated 11-deoxycortisol with the microorganism Rhizopus nigricans, one of the conversion products isolated and identified was 11α, 17α,21-trihydroxyallopregnane-3,20-dione. The incubation of progesterone with Cunninghamella blakesleiana (134) demonstrated the conversion to 16α-hydroxy-pregnane-3,20-dione indicating that reduction to both ring A reduced stereoisomers is possible in microbiological systems.

The reduction of the ring A double bond appears to be essentially an irreversible reaction, since the formation of the C-4(5) double bond has never been demonstrated to take place in the mammalian organism. The possibility of such a reaction cannot be excluded since desaturation reactions have been shown to take place in vitro. The formation of the Δ¹ and the Δ₄ double bond has been shown in vitro for microorganisms (215). Meyer (125) recently proved the conversion
of 19-hydroxy-\(\Delta^4\)-androstene-3,17-dione to estrone in homogenates of human placenta, and Baggett et al. (4) isolated and identified radioactive estradiol-17\(\beta\) by the radioisotope dilution technique from the incubation of radioactive testosterone with human ovarian tissue.

Other \(\Delta\)-hydrogenase reactions have also been demonstrated. Saturation of \(\Delta^1\)-androstene-3,17-dione in a human hypogonadal male (209) and of \(\Delta^1,\Delta^4\)-androstadiene-3,17-dione in a human male with rheumatoid arthritis (59) have been observed by isolation and identification of the reduced products in the urine. The existence of the \(\Delta^{16}\)-hydrogenase has been shown by Dorfman et al. (59). These workers isolated from urine pregnane-3\(\alpha\),20\(\alpha\)-diol after the administration of \(\Delta^{16}\)-dehydroprogrenolone to a human female and \(\Delta^{16}\)-dehydroprogesterone to post menopausal women. Neither the \(\Delta^1\) nor the \(\Delta^{16}\)-hydrogenase reactions have been shown to occur in mammalian tissue in vitro. The \(\Delta^1\)-hydrogenase reaction can be effected by yeast cells (32) and the \(\Delta^{16}\)-hydrogenase reaction by preparations of the mold, \textit{Rhizopus arrhizus} (131).
ACTION OF 3α AND 3β-DEHYDROGENASES

Fig. 6. Reversible reduction of the C-3 keto group.

Closely associated with hydrogenation of the C-4(5) double bond in the biologically active steroids is reduction of the C-3 ketone group to the 3α and 3β-hydroxyl forms. This has been observed to take place in vivo in a number of animal species with a variety of substrates. In humans the reduction proceeds mainly to the 3α form (60). A greater amount of 3β-hydroxy steroids seems to be excreted in the rodent (30,181).

In in vitro systems of liver slices (96,173,184), homogenates (201,203,208) and perfusion through intact livers (38,37,36) in which a variety of steroids have been studied both the 3α and 3β-hydroxy forms have been isolated and identified. When Schneider (184) incubated deoxycorticosterone with rat liver slices he obtained conversion predominantly to
the 3β-hydroxy derivatives. Contrarywise, Ungar and Dorfman (208) incubating 17α,21-dihydroxyprogesterone-3,20-dione with a rat liver supernatant obtained the 3α-hydroxy epimer as the major conversion product. Ungar (205) has studied the steric course of the reduction of the C-3 ketone of C19 and C21 reduced ring A, 3-keto compounds using rat and chick liver supernatant and residue fractions. In the C19 series, those steroids containing the 17-ketone or 17-ketone forming group (androstanolone, androstane-3,17-dione, etiocholanolone and etiocholane-3,17-dione) yielded the 3β-hydroxy isomer predominantly when incubated with the supernatant fraction. The 17-deoxy C21 compounds (21-hydroxyallopregnane-3,20-dione, pregnane-3,20-dione) also yielded mainly the 3β-hydroxy isomers. The 17α-hydroxy C21 steroids (17α,21-dihydroxyprogesterone-3,11,20-trione, 11β,17α,21-trihydroxyprogesterone-3,20-dione and 17α,21-dihydroxyprogesterone-3,20-dione) yielded the 3α-hydroxy isomer when incubated with the supernatant fraction. The latter compounds also gave rise to the 3α-hydroxy isomer when incubated with the residue fraction. In contrast, the C19 steroids and C21 deoxy compounds were reduced to the 3α-hydroxy isomers predominantly in the residue fraction. Rubin (166) has made the interesting observation that the sex of the animal may have influence on the steric course of the reduction at C-3. In a series of experiments this worker has noted consistent and significant sex differences in the ratio of 3β-hydroxy to 3α-hydroxy products when 3-keto C19 androgens were incubated
with homogenates of livers obtained from males and those obtained from female rats. Incubation of androstane-3,17-dione and \( \Delta^4 \)-androstene-3,17-dione with male rat livers yielded a ratio of 2.5 whereas with female rat liver the ratio was 0.25. Furthermore, incubation with livers from castrated male rats resulted in an 0.25 ratio, the same as that obtained for females. However, livers from castrated male rats that had been treated with testosterone, produced a ratio again of 2.5. Incubation of testosterone with livers from normal male rats yielded the 2.5 ratio whereas when the same substance was incubated with livers from female rats, reduction at C-3 proceeded only to the 3α-hydroxy compound, no 3β-reduction occurred. It should be noted that this study was carried out only with C_{19} compounds as substrates and whether this sex difference would obtain with C_{21} steroids, has not been tested. Horwitt and Segaloff (96) reported the isolation of only pregnane-3α,20α-diol after the incubation of progesterone in rabbit liver slices. This has been recently confirmed by Taylor (201) who studied the metabolism of progesterone in rabbit liver homogenate. He isolated and identified several metabolites and found that reduction to the 3α-hydroxy isomer exceeded by far the reduction to the 3β-hydroxy form. In the case of Tomkins and Isselbacher (203) employing a highly purified supernatant from a rat liver homogenate, they were able to isolate only the 3α-hydroxy derivative of cortisone. These conflicting data may be due to the type of tissue
preparation employed, the nature of the side chain on the steroid molecule employed as substrate, a true species difference or incomplete isolation of metabolites.

The dehydrogenase reaction, which involves oxidation of the secondary alcohol group to the ketone function, has been observed in vivo. Schiller and Dorfman (181) isolated and identified epiandrosterone from the urine of guinea pigs receiving androsterone and Dorfman et al. (58) were able to isolate androsterone from the urine of a hypogonadal male receiving epiandrosterone. Ungar et al. have shown the conversion of androstane-3β,17β-diol to androsterone (210) and Δ⁵- androstene-3α,17β-diol (212) to androsterone and etiocholanolone. Schneider (183) isolated the dihydropregnane derivative of cortisone from normal human urine and suggested that this compound may have been formed by reoxidation of the C-3 hydroxyl group of the tetrahydropregnane derivative of cortisone (urocortisone). This conclusion would seem to be supported by the observation of Savard and Goldfaden (179) who tentatively identified the dihydropregnane derivative of cortisol among the urinary metabolites of the orally administered tetrahydropregnane derivative of cortisol (urocortisol) in a patient. Schneider and Mason (185) isolated androstane-3,17-dione and etiocholane-3,17-dione after incubating androsterone and etiocholanolone respectively with rabbit liver slices. Further evidence for the reaction was afforded by Kochakian and Aposhian (102) who found androstane-3,17-dione as one of the transformation products of androstane-3α,17β-diol
incubated with rabbit liver tissue. Interconversion of the two possible stereoisomers can occur presumably by way of a 3-keto intermediate. Schneider and Mason (185) incubated androsterone with rabbit liver slices and isolated epiandrosterone as a metabolite, and Kochakian and Aposhian (102) obtained epiandrosterone from androstane-3α,17β-diol in the presence of rabbit liver slices.

Tomkins and Isselbacher (203) studied the conversion of the dihydropregnan derivative of cortisone to uro-cortisone spectrophotometrically by measuring the absorption of the reduced and oxidized pyridine nucleotide at λ 340 μ and showed the reaction to be readily reversible with the equilibrium toward the reduced (tetrahydro) product. The enzyme system required either DPN or TPN and was completely inhibited by p-chloromercuribenzoate. The inhibition could be reversed by glutathione. Ungar and Dorfman (208) have described a 3-hydrogenase in rat liver. They found that the major activity resided in a cell free supernatant of a pH 7.4 buffer extract of rat liver ground in a meat grinder. The activity was retained in the supernatant of an acetone powder of the ground rat livers. Dialysis of the supernatant lowered the conversion of the 3-ketone of 17α,21-dihydroxypregnane-3,20-dione to 30 percent but the addition of 0.002 M DPN increased the conversion to 95 percent.

Talalay and Marcus have described the purification and properties of a 3α-hydroxysteroid dehydrogenase from cell free extracts of a pseudomonas species (199). This preparation
catalyzed freely reversible interconversions of the type:

\[
\text{Androsterone} + \text{DPN}^+ \rightleftharpoons \text{Androstane-3,17-dione} + \text{DPNH} + H^+
\]

They claim to have effected a 30-50 fold purification over the original extracts and a virtual separation from a \(\beta\)-hydroxy steroid dehydrogenase. The enzyme activity was measured spectrophotometrically by observing the rate of appearance of the DPNH absorption band at 340 \(\text{nm}\) or the rate of disappearance of the DPNH absorption band. Free sulfhydryl groups appeared necessary for enzyme activity inasmuch as the enzyme was strongly inhibited by mercuric ion, \(p\)-chloromercuribenzoate, ferrous ion and cupric ion. Specificity studies indicated that the 3\(\alpha\)-hydroxy groups of both the androstane or etiocholane series were oxidized but that other hydroxyl groups in positions such as 3\(\beta\), 6\(\beta\), 11\(\alpha\), 11\(\beta\), 16\(\alpha\), 17\(\alpha\), 17\(\beta\) and 21 were not oxidized. Conversely only 3-ketosteroids were reduced by the enzyme to the corresponding 3\(\alpha\)-hydroxy-steroids. If the 3-keto group was associated with either a \(\Delta^1\) or \(\Delta^4\) double bond, the compound was not reduced.

Talalay and Dobson have described the preparation and purification of a \(\beta\)-hydroxysteroid dehydrogenase from cell free extracts of a pseudomonas species (198) which has been shown to catalyze the following reactions:

1) Dehydroepiandrosterone + DPN⁺

\[
\Delta^4\text{-androstene-3,17-dione} + \text{DPNH} + H^+
\]
2) \(\Delta^5\)-androstene-3\(\beta\),17\(\beta\)-diol + 2 DPN⁺

\[
\Delta^4\text{-androstene-3,17-dione} + 2\text{DPNH} + 2H^+
\]
3) Epiandrosterone + DPN$^+$

\[
\text{androstane-3,17-dione} + \text{DPNH} + \text{H}^+\]

The enzymes appeared to be steroid specific and required sulfhydryl groups for activity. 3α and 17α-hydroxy steroids were not oxidized by the enzyme, nor did they inhibit the oxidation of their β-epimers. The 3β and 17β-oxidizing capacity appeared to be attributable to the same enzyme since no separation of these activities was achieved.

ACTION OF THE 11β-DEHYDROGENASES

Fig. 7. Reversible reduction of the C-11 keto group.

In vivo reduction of the 11-keto group of administered cortisone (29) by isolation of urinary cortisol and that of adrenosterone (177) by the isolation of 11β-hydroxy-androsterone and 11β-hydroxyetiocholanolone has been reported. This reaction has been found to be reversible for cortisone and cortisol as well as other corticosteroids such as 11-dehydrocorticosterone and corticosterone which have been shown to be interconvertible by in vivo techniques (151).
In vitro, reduction of cortisone to cortisol has been shown by Caspi et al. (38) and Fish et al. (65). Both groups of workers were able to isolate and characterize the product. Amelung and co-workers (2) have observed that the enzymatic mechanism (for conversion of cortisone to cortisol) appeared to be associated with liver microsomal fraction and not the mitochondria. They also noted that the conversion of cortisone to cortisol was oxygen dependent and facilitated by addition of DPN, Mg++, ascorbic, citric and succinic acids. The reverse reaction of cortisol to cortisone has not yet been demonstrated in vitro.

Reduction of the 11-ketone to the 11α-hydroxyl group has not been demonstrated either in vitro or in vivo for the mammalian organism. This finding is in harmony with isolation studies of adrenal tissue which thus far have not revealed any 11α-hydroxylated steroids.

ACTION OF THE 16α and 16β-DEHYDROGENASES

Fig. 8. Reduction of the C-16 keto group
The reduction of 16-keto estrone and 16-keto estradiol-17β to the corresponding 16α-hydroxy derivatives has been demonstrated in vivo in a human male (191). The metabolite estriol was identified on the basis of colorimetric evidence. Evidence has not been presented for the reaction taking place in vitro, nor has anything been reported on either the in vivo or in vitro reduction of the 16-ketone to the 16β-hydroxy form. One 16β-hydroxy steroid, epiestriol, has been isolated from human pregnancy urine (120).

**ACTION OF THE 17β AND 17α-DEHYDROGENASES**

![Fig. 9. Reversible reduction of the C-17 keto group.](image)

The conversion of the C-17 carbonyl to the 17β-hydroxyl group has been documented from in vivo studies in which etiocholane-3α,17β-diol was isolated from urine after the administration of etiocholane-3α,17-one to a human male (72). Similarly dehydroepiandrosterone (128,122) was
metabolized to 17β-hydroxy products. In the estrogen series, Pearlman and Pincus (139) established the conversion of estrone to estriol in a human male. The isomeric reaction, conversion of the 17-ketone to the 17α-hydroxyl product has not been demonstrated \textit{in vivo} when the C$_{19}$-17-ketosteroids were administered. However, in the C$_{18}$ or estrogen series, estrone administration to rabbits resulted in the isolation of estradiol-17α (193) and the same metabolite was characterized from the bile of a dog receiving estrone (133). Interconversion of estradiol-17β and estradiol-17α has been shown to occur \textit{in vivo} for the rabbit (67).

\textit{In vitro} rabbit liver slices have been shown to convert androsterone and etiocholane-3α-ol-17-one to the 17β-hydroxy derivatives (185). Schneider and Mason (186) and Rosenkrantz and Dorfman (160) incubated dehydroepiandrosterone with rabbit liver slices and homogenate respectively and also showed conversion to the 17β-hydroxy derivatives. Similar results were observed by Ungar et al. (212) using a rat liver homogenate. The conversion of estrone to estradiol-17β by rat liver (169) and various human tissues (168) has also been reported. Reduction to the 17α-epimer has been demonstrated in rabbit liver slices by Clark and Kochakian (39) and Schneider and Mason (185). In the former study $Δ^4$-androosterone-3,17-dione was converted to epitestosterone and in the latter etiocholanolone was converted to the 3α,17α-diol.

Rosenkrantz (161) incubating rabbit liver homogenate with
dehydroepiandrosterone also isolated and identified the 17α-hydroxy derivative.

The reverse reaction, oxidation of the 17-hydroxyl group to the 17-ketone has been well documented from in vivo studies for the 17β-hydroxy compounds. The 17-keto derivatives of testosterone were isolated from urine after the administration of the latter hormone to a human male (51, 34, 48, 72), human female (182), pregnant monkey (95) and the male guinea pig (52). Similarly the conversion of estradiol-17β to estrone has been shown in vivo for male and female guinea pigs (69, 68), the female rabbit (67) the pregnant monkey (61) and a human male (86).

Clark and Kochakian (39), using in vitro techniques, demonstrated that rabbit liver homogenates oxidized 17β-hydroxy steroids to the corresponding 17-ketosteroids. Kochakian and Aposian (102) extended the studies to guinea pig liver and kidney homogenates which were also shown to carry out the oxidation.

Kochakian et al. (103) have been the only workers to report the oxidation of the 17α-hydroxyl to the 17-ketone function in rabbit liver tissue in vitro.

Sweat et al. (195) have described the concentration of a DPN-linked dehydrogenase responsible for the formation of the C-17 carbonyl from the C-17 hydroxyl compound. The enzyme was found in the supernatant after centrifugation of a liver homogenate for one hour at 20,000 x g and was
precipitated by full saturation with ammonium sulfate. When such preparations were incubated with testosterone the only product formed was Δ⁴-androstene-3,17-dione. As described above, Talalay and Dobson (198) have studied the properties of a β-hydroxysteroid dehydrogenase from cell free extracts of a pseudomonas species capable of reversibly oxidizing the 17β-hydroxyl group to the 17-ketone.

**ACTION OF THE 20β AND 20α-DEHYDROGENASES**

![Diagram](image)

**Fig. 10.** Reversible reduction of the C-20 keto group.

The C-20 ketone function in the C₂₁ steroids can also undergo reduction resulting in two possible isomeric forms, the 20β-hydroxy and the 20α-hydroxy compounds. In *in vivo* human metabolism studies, the reduction appears to be specifically directed to the 20α-hydroxy form (60). However, Burstein and Dorfman (24) administered cortisol orally to
guinea pigs and isolated from urine both the 20α and 20β-hydroxy derivatives of cortisol.

**In vitro** experiments utilizing homogenates of bovine corpora lutea, Hayano et al. (81) observed the C-20 reduction of progesterone and deoxycorticosterone to the 20β-hydroxy derivatives. Caspi et al. (38) perfused cortisone through rat livers and isolated the 20β reduced derivative as one of the conversion products. Similarly, 21-hydroxypregnane-3,20-dione and 3α-hydroxypregn-20-one was observed to be reduced to the 20β-hydroxy derivatives after incubation with a chick liver homogenate supernatant (207). Schneider (184) incubating deoxycorticosterone with rat liver slices was unable to determine the configuration of the hydroxyl group of the 20-reduced metabolite. That the reaction does proceed to the 20α-hydroxy derivative **in vitro** has been reported by Hübener and Schmidt-Thome (97) who isolated the 20α-hydroxy derivative of 11-deoxycortisol after incubating the latter with a rat liver homogenate. The same results were obtained by Hayano (77), in the incubation of 11-deoxycortisol, progesterone and deoxycorticosterone with pig liver homogenates. Progesterone incubated with rabbit liver slices (96) and homogenates (201) also yielded 20α-hydroxy reduced products.

The reverse reaction, oxidation of the 20α or 20β-hydroxyl group has never been demonstrated **in vivo**. However, Grant (74) did provide evidence for the **in vitro** conversion of pregnane-3α,20α-diol in rat liver tissue to pregnan-3α-ol-20-one.
Recknagel and Glen (148) have studied the properties of an enzyme system in rat liver homogenates which presumably altered the side chain of cortisone under anaerobic conditions by reduction of the C-20 ketone to a C-20-hydroxy group. The sulfuric acid-phenylhydrazine reaction of Porter* and Silber (142) was used to measure alterations of the 17,21-dihydroxy-20-keto side chain. They found that 70 percent of the activity of the original homogenate could be accounted for in the microsome and supernatant fraction. Mitochondria had little or no activity and the cell nuclei were completely inactive. The enzyme system had a sharp optimum in the region of pH 7.4-8.0 and TPNH was suggested as a necessary cofactor. These workers made no attempt to characterize the conversion products.

**ACTION OF THE DESMOLASES**

The desmolase reaction involving rupture of the carbon-carbon bond has been shown to take place between carbon atoms 20 and 21 yielding the corresponding etio acid (Fig. 11,a). This reaction has only been demonstrated by perfusion. Picha et al. (136) perfused deoxycorticosterone through the intact rat liver and Levy (110) perfused the same compound through the intact adrenal gland and were able to isolate and identify \( \Delta^4 \)-etiocholenic acid as one of the conversion products.
Fig. 11. Rupture of carbon-carbon links (desmolases)
A second desmolase reaction involves rupture between carbons 17 and 20 to yield C₁₉ compounds (Fig. 11,b). This latter reaction has been widely demonstrated in vivo and only those steroids possessing the 17α-hydroxy group appear to be transformed to 17-ketosteroids. This has been shown to be the case with patients receiving 11-deoxycortisol (11, 12,9) and its dihydro-5β derivative (207). The major urinary 17-ketosteroid metabolites of the 11-oxy-17-hydroxycortico-steroids appeared to be 3α,11β-dihydroxyetiocholan-17-one and 3α-hydroxyetiocholane-11,17-dione. The conversion products have been demonstrated in urine of patients given cortisone (10,29,47,12), cortisol (27,47), dihydrocortisol-5β and uro-cortisol (179) and in one of two patients receiving 21-deoxy-cortisone (28). In addition the Δ⁹(11) dehydration artifact of the 11β-hydroxy metabolites formed during hot acid hydrolysis has been frequently noted (10,29,27,179).

In only one study with surviving tissue has the isolation and characterization of the 17-ketosteroid products from a C₂₁ steroid been accomplished. Caspi et al. (38) and Caspi and Hechter (37) have succeeded in isolating and identifying adrenosterone and androstane-3α-ol-11,17-dione when cortisone was perfused through isolated rat livers. The reverse reaction, synthesis of C₂₁ compounds from C₁₉ compounds has yet to be conclusively demonstrated either in vivo or in vitro.

Certain species of molds have the ability to
rupture the carbon-carbon bond between 17 and 20 irrespective of the presence or absence of the 17α-hydroxy group. This has been proven in the conversion of progesterone to Δ⁴-androstene-3,17-dione and its 6β-hydroxy derivative and deoxy­corticosterone to Δ⁴-androstene-3,17-dione (135). Since the hydroxylation of steroid hormones has been shown to readily take place in the presence of various species of microorgan­isms, it is likely that 17α-hydroxylation in these instances preceded side chain removal.

A third desmolase reaction (Fig. 11,c) involving rupture of the bond between carbons 20 and 22 of the choles­terol side chain has been reported by Saba et al (170) and Lynn et al. (118). Both groups were able to demonstrate the \textit{in vitro} conversion of cholesterol to pregnenolone and pro­gesterone in bovine adrenal homogenate preparations.

A fourth desmolase reaction has been recently re­ported involving removal of carbon 19 from the steroid nucleus (Fig. 11,d). Meyer (125) incubated 19-hydroxy-Δ⁴-androstene-3,17-dione with human placenta and demonstrated the conversion to estrone and estradiol-17β and Baggett et al. (4) were able to show the conversion of testosterone to estradiol-17β after incubation with human ovarian tissue.

No definitive studies have appeared regarding the properties of the enzyme systems involved in the rupture of the carbon to carbon links in the steroid molecule.
ACTION OF THE DEOXYLASES

The reduction of the C-21 primary alcohol group to a methyl group (Fig. 12,a) has been well documented in vivo but still remains to be demonstrated in any in vitro system. It has been shown that deoxycorticosterone is transformed by the rabbit (92), chimpanzee and the human male (131) to the 21-deoxy metabolite, pregnane-3α,20α-diol. Other reports have shown the reaction to take place after the administration of 11-dehydrocorticosterone to a man and woman (121), cortisone to a man and woman (47) and 17α,21-dihydroxypregnane-3,20-dione to a rheumatoid arthritic patient (206). The latter two conversions are illustrated in Fig. 12,b.

Evidence has been presented for the conversion of the 17-hydroxy C_{21} steroids to 17-deoxy C_{21} steroids, that is removal of the 17α-hydroxy group without rupture of the side chain. Ungar et al. (206) after administering 17α,21-dihydroxypregnane-3,20-dione to postmenopausal women, were able to show an increased excretion of pregnane-3α,20α-diol (Fig. 12,c). Similar results were reported by Rossalet et al. (164). After administration of 21-deoxycortisol and 17-hydroxyprogesterone to patients they observed in both instances an increased excretion of 17-deoxy C_{21} steroids in urine (Fig. 12,d).

Miller and Axelrod (129) perfused cortisone through cirrhotic rat livers and tentatively identified 6β-hydroxydeoxycorticosterone from extracts of the perfusates. This conversion, if confirmed would be remarkable since this would indicate the reductive removal of the oxygen at C-11, a
Fig. 12. Reduction of hydroxyl groups to hydrocarbon residues (deoxylases)
reaction thus far not demonstrated.

ACTION OF THE HYDROXYLASES

![Chemical structure diagram]

**Fig. 13.** Hydroxylation of the steroid nucleus.

The isolation of 3α,6α-dihydroxyallopregnan-20-one by Dobriner et al. from pregnancy urine (112) provided evidence for the possibility for 6-hydroxylation occurring in vivo. This was followed by the isolation of 3β,6α-dihydroxyallopregnan-20-one from human urine by Salamon and Dobriner (171). Burstein and Dorfman (25) isolated 6β-hydroxycortisol from human urine during late pregnancy and after the administration of cortisol to a patient with Cushing's disease following surgical removal of the adenomatous adrenal. The same steroid has also been isolated from the urine of normal and scorbutic guinea pigs (26).

By in vitro methods 6-hydroxylation has been observed with adrenal tissue (75,106), corpus luteum (82) and a variety
of microorganisms (131). The predominant form isolated from mammalian species has been the 6β-hydroxy compound. However, Meyer et al. (126) using adrenal homogenates were able to isolate and identify both the 6α and 6β-hydroxy epimers. Axelrod and Miller (3) have indicated the presence of the 6β-hydroxylating enzyme in rat liver tissue. They perfused deoxycorticosterone, testosterone and Δ⁴-androstene-3,17-dione through intact cirrhotic rat livers and claimed to have identified the respective 6β-hydroxylated derivatives. The characterizations were made on the basis of color reactions, ultraviolet absorption and sulfuric acid chromogen spectra and mobility rates on paper.

16α-Hydroxylation has been demonstrated in vivo in a case of Addison’s disease. The two hydroxylated steroids etiocholane-3α,16α,17β-triol and androstane-3α,16α,17β-triol were isolated from human urine by Lieberman et al. (114). 16α-Hydroxylation was demonstrated by in vitro methods in rabbit liver slices by Schneider and Mason (186). The metabolite Δ⁵-androstene-3β,16α,17β-triol was isolated after the incubation of dehydroepiandrosterone. The same reaction has been shown to take place in the presence of actinomycoses (135), progesterone being converted to its 16α-hydroxy derivative.
CHAPTER IV

11-DEOXYCORTISOL
Reichstein first isolated 11-deoxycortisol from beef adrenal gland extracts in 1936 (149) and assigned to the compound the letter designation "S". This compound was isolated as the acetate and, interestingly enough, it had the same melting point as cortisone acetate. Mixed melting points of the two compounds produced no depression, but a difference was detected in the elementary composition in addition to differences in color reactions when the steroids were treated with concentrated sulfuric acid. Cortisone gave an orange color with a green fluorescence, while 11-deoxycortisol gave a pure red color. Oxidation with chromic acid yielded the C19 steroid Δ⁴-androstene-3,17-dione.

The structure of 11-deoxycortisol was established by partial synthesis (Fig. 14) from dehydroepiandrosterone (I) by von Euw and Reichstein (216). The reaction of I with allylmagnesium bromide yielded the 17β-hydroxy-17α-allyl derivative (II) which after an Oppenauer oxidation was transformed to 17α-allyltestosterone (III). Dehydration of the tertiary alcohol with phosphorous oxychloride in pyridine gave a triene (IV). The two side chain double bonds were hydroxylated with osmium tetroxide to produce the tetrol (V). Following protection of the C-21 and C-22 hydroxyl groups by acetonide formation (VI), the C-20 monoacetate (VII) was obtained on acetylation with acetic anhydride in pyridine. The acetonide complex was then decomposed by treatment with acetic acid and oxidation of the resulting glycol (VIII) with periodic acid yielded the acetoxy
Fig. 14. Partial synthesis of 11-deoxycortisol from dehydroepiandrosterone
aldehyde \((IX)\). The free dihydroxy aldehyde \((X)\) obtained on gentle saponification, rearranged in hot pyridine to the dihydroxy acetone grouping forming 11-deoxycortisol \((XI)\).

The biological activity of 11-deoxycortisol, as far as is known, appears to be quite limited. On the other hand, since the compound does possess certain of the prerequisite structural features necessary for adrenocortical activity (\(\Delta^4\)-3-ketone group, 17\(\alpha\)-hydroxyl group and an \(\alpha\)-ketol side chain) its function as a precursor to more active compounds deserves very serious consideration. Hydroxylation at C-11 would result in the biosynthesis of the highly active cortisol, whereas oxidative removal of the side chain, would result in C\(_{19}\) compounds possessing androgenic activity.

Evidence for the biosynthesis of 11-deoxycortisol \emph{in vitro} has been presented by Brady et al. (19) who incubated acetate-\(C^{14}\) with dog adrenal slices and by Haines et al. (75) incubating acetate-\(C^{14}\) with hog adrenal brei in the presence of ACTH. In both instances characterization was based on paper chromatographic behaviour. Zaffaroni et al. (230) perfused acetate-\(C^{14}\) through beef adrenals and were not able to detect the presence of 11-deoxycortisol in the perfusate. On the other hand Kushinsky (106) did isolate and identify 11-deoxycortisol from perfusates of progesterone through beef adrenal glands.

Indirect evidence has been obtained for the endogenous secretion of 11-deoxycortisol in humans, since recently
two independent groups of workers (165, 204) isolated and identified 3α,17α,21-trihydroxypregn-20-one, a reduced metabolite of 11-deoxycortisol, from the urine of patients. Dobriner (47) has suggested the possibility of 11-deoxycortisol being a precursor of urinary androsterone and etiocholanolone, since the compound has no oxygen function at C-11 and it is present in adrenal gland extracts. Birke (9) tested Dobriner's hypothesis by administering large doses of 11-deoxycortisol acetate by mouth to patients. The result was a large increased excretion of 17-ketosteroids which consisted mainly of etiocholanolone and very small amounts of androsterone.

The role of 11-deoxycortisol, as a precursor of cortisol, has been well established by in vitro methods. 11-Deoxycortisol incubated with beef adrenal homogenates (80, 124, 180), hog adrenal bries (75) and perfused through the isolated beef adrenal gland, (91) has been shown to be converted to cortisol. Hayano and Dorfman were able to effect the conversion (11-deoxycortisol to cortisol) with a beef adrenal homogenate residue (80) and Sweat reported (194) that all the enzymic activity necessary for the transformation of 11-deoxycortisol to cortisol resided in the cell granules, presumably the mitochondrial fraction which was sedimented at 19,000 x g. None of this activity could be found in the supernatant or loosely packed layer. The conversion has been extended by Murray and Peterson (131) and Collingsworth et al. (42) to
include microbiological systems.

Reichstein and Shoppee (150) have reported that 11-deoxycortisol was active in the Everse-de Fremery test in which the recovery of the fatigued muscle and survival in adrenalectomized rats was measured. This test correlates well with the electrolyte activity of a compound. However, the compound proved to possess very little or no activity in Ingle's work-performance test in adrenalectomized rats (98) which is correlated with carbohydrate activity. Clinton et al. (41) studied the effect of 11-deoxycortisol on the renal excretion of electrolytes in a normal dog and concluded that 11-deoxycortisol belonged to the group of compounds possessing sodium and chloride retaining properties. Apparently modification of the deoxycorticosterone molecule by introducing a hydroxyl group at C-17 depressed the sodium and chloride retaining properties. Masson et al. (123) made a comparative study of the activities of 11-deoxycortisol and deoxycorticosterone and observed the former compound to be approximately one-thirteenth as active as the latter in the survival of adrenalectomized rats, but 11-deoxycortisol acetate did not affect diuresis as did deoxycorticosterone acetate. They also observed that in unilaterally nephrectomized rats fed a high sodium diet, 11-deoxycortisol did not enhance or inhibit the hypertension produced by deoxycorticosterone acetate. The latter result is in disagreement with the observation of Selye (189) who found that 11-deoxycortisol did produce hypertension if given in
adequate doses. Fajans et al. (64) investigated the metabolic effects of 11-deoxycortisol in two normal men and found no effect on electrolyte, water or "organic" metabolism. The relationship of structure of adrenocortical steroids to anti-phlogistic activities was studied by Dougherty and Schneebell (62). Using the loose connective tissue spread method, they found that 11-deoxycortisol did possess some antiphlogistic activity but at much higher dose levels than the more active adrenocortical steroids. Kochakian and Robertson (104) found that 11-deoxycortisol had no effect on the body composition of mice with respect to protein and fat, and Kochakian reported that the compound had no influence on body, thymus and spleen weights in rats.
CHAPTER V

METHODS
INCUBATION MEDIUM

Medium Employed in Part I

For each 5 mg. of steroid incubated, the following were employed:

- Glutamate 0.16M 1.0 ml.
- Nicotinamide 0.16M 0.50 ml.
- Phosphate buffer 0.10M 3.5 ml.
- Diphosphopyridine nucleotide (DPN) 0.02M 1.0 ml.

L (+) glutamic acid was dissolved in 10 per cent KOH to pH 7.0, while the phosphate buffer at pH 7.35 was prepared from 0.1M Na₂HPO₄ and 0.1M NaH₂PO₄. The DPN was neutralized before use. The steroid was dissolved in propylene glycol (2.5 mg./0.15 ml.) with the aid of ether which was removed under vacuum once solution was effected.

Medium Employed in Part II

A Krebs-ringer-phosphate buffer was used as the incubation medium for the ultracentrifugation studies and was prepared as follows:

<table>
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<th>Component</th>
<th>Molarity</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
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<td>100 parts</td>
<td></td>
</tr>
<tr>
<td>0.154M KCl</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.11M CaCl₂</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.154 KH₂PO₄</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.154 MgSO₄</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.10M Phosphate buffer pH 7.35</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
PREPARATION OF TISSUE

Male and female albino rats, weighing approximately 300 g., were sacrificed by decapitating and bled. The livers were removed within one minute and placed on ice, and homogenized at 5° C. in a Waring blender for 30 seconds. The homogenate was strained through gauze to remove connective tissue and stored at -10° for not longer than two hours. In Experimental-Part I, the strained homogenate was centrifuged at 6000 x g. for 30 minutes in a Servall angle head centrifuge using an SS-1 rotor. The residue was discarded and the supernatant used as the tissue preparation for the incubations. In Experimental-Part II, the homogenate was first centrifuged in the same manner as above to obtain a 6000 x g. residue and a supernatant. The supernatant was further centrifuged in a Spinco Model L ultracentrifuge at 78,000 x g. for 90 minutes to obtain a 78,000 x g. residue and a particulate free supernatant.

INCUBATION PROCEDURE

Suitable aliquots of the steroid solution (equivalent to 10 mg. of steroid) were placed in 125 ml. Erlenmeyer flasks followed by the tissue preparation (20 ml.) and the DPN solu-
tion. The flasks were rotated to insure thorough mixing of the contents and placed in a Warburg bath, with the flasks open to the atmosphere at 38° C. for two hours.

EXTRACTION PROCEDURE

The contents of the incubation flasks were added to 10 volumes of freshly distilled acetone. This afforded precipitation of the proteins and solution of the steroids into the acetone. The acetone mixture was stirred overnight at 5° C. and then filtered through a Buchner funnel followed by twice washing of the residue remaining on the funnel with hot acetone. The filtrates were combined and concentrated in vacuo to remove the acetone. The removal of fats was accomplished by adjusting the residual aqueous phase to approximately 70 per cent methanol and extracting with an equal volume of ligroin. The extraction procedure was repeated twice more with fresh portions of ligroin. The ligroin fractions were pooled and washed with one-half volume of 70 per cent methanol. The aqueous methanol fractions were then combined and the methanol removed by distillation in vacuo, the distillation flask being rotated frequently to prevent superheating and bumping.

After removal of the methanol, the aqueous phase was extracted three times with equal volumes of chloroform and the aqueous residue discarded. The chloroform extracts were combined, washed two times with approximately one-twentieth volume of distilled water and dried overnight over
sodium sulfate. Finally, the chloroform extract after de­cantation from the sodium sulfate was concentrated in vacuo, transferred to a tared vessel, partially dried in a stream of nitrogen at 35-40° C. and completely dried in a vacuum dessicator.

PURIFICATION PROCEDURES

Column Adsorption Chromatography

Column adsorption chromatography was employed as an initial purification procedure. In general, the tube diameter was selected so that the height of the silica gel was approximately three times the diameter. Slurries of silica gel (Davidson, 100-200 mesh) in benzene were transferred to a suitable glass chromatography tube containing a glass wool plug. The silica gel was permitted to pack by gravity until a column was formed. The extract to be chromatographed was dissolved in a minimum volume of benzene and deposited carefully on the upper surface of the column. Elution was carried out with benzene, benzene-ethyl acetate mixtures (19:1, 14:1, 9:1, 5:1, 4:1, 3:1, 2:1 and 1:1), ethyl acetate and finally methanol. The eluates were collected in round-bottom flasks and concentrated in vacuo. The concentrates were then transferred to small tared tubes and thoroughly dried and weighed.

Paper Partition Chromatography

Further resolution of substances derived from the
incubation extracts, which were initially fractionated on a silica gel column, was obtained by paper partition chromatography. Three different systems were used: 1) the toluene-propylene glycol system (31) for the resolution of steroids of intermediate mobility rates, 2) the ligroin-propylene glycol system (176) for the resolution of fast moving compounds such as the C_{19}-17-ketosteroids and finally the chloroform-formamide system (227) for the resolution of very polar or slow moving compounds. Whatman #1 filter paper was divided into 16-17 x 58 cm. strips which were put through a preliminary washing with benzene-ethanol mixture (1:1) for 48 hours in a Soxhlet extractor and dried at room temperature. The dried, washed strips were impregnated with a 1:1 mixture of methanol and propylene glycol or a 1:1 mixture of methanol and formamide in the case of the chloroform-formamide system, blotted, and the material to be chromatographed was applied along a penciled line 10 cm. from one end of the paper strips. The application of the extract was performed by dissolving the material in a 1:1 mixture of methanol and methylene dichloride and depositing the solution along the start line with intermittent drying of the solvent in a nitrogen stream. Ordinarily, the papergrams were developed in a particular system for from 10-72 hours. A run-off beaker was placed at the bottom of the chromatograms to collect any steroid material that may have run off the paper with the effluent. The chromatographic strips were dried in
a hood at room temperature overnight.

For locating the various substances, 2 mm. strips were cut from the chromatogram and exposed to several color-forming reagents. This procedure permitted positioning of the steroids and subsequent separation of the individual components on the papergrams. Those parts of the paper containing compounds as detected by the color reactions were cut up into small pieces and eluted with methanol-methylene dichloride (1:1) for 24 hours. This period of time was sufficient for complete elution. The eluates from the paper were filtered through absorbent cotton into round-bottom flasks, the solvent being removed by vacuum distillation, followed by several additions of benzene and redistillation to remove traces of stationary solvent (propylene glycol or formamide) that may have remained on the paper. Complete removal of any stationary solvent present was assured by further drying of the paper eluates in high vacuum (0.001-0.003 mm. Hg.).

**Digitonin Separation**

The following procedure was used to separate the 3α-hydroxy and 3β-hydroxy stereoisomers. A 2 per cent solution of digitonin in 90 per cent ethanol was added to an equal volume of a solution of the steroid in 90 per cent ethanol and permitted to remain at room temperature overnight. A digitonin-steroid ratio of 5:1 was used. The formation of a precipitate revealed the presence of a 3β-hydroxy compound while any 3α
component present remained in solution. The precipitate was centrifuged, washed successively with 90 per cent ethanol and ether and dried. The 3α fraction and the alcoholic and ether washings were combined and evaporated to dryness in a stream of nitrogen.

The digitonide complex was decomposed by dissolving in warm pyridine and allowing the solution to stand overnight at room temperature. After this period of time the addition of ten volumes of ether precipitated the digitonin which was centrifuged and washed three times with ether. The ether fractions were combined, washed successively with 2N HCl, 1N NaOH, and finally with distilled water until neutral. The aqueous washings were extracted twice with ether. The ether fractions were combined, dried overnight with sodium sulfate, decanted and evaporated to dryness in vacuo.

ANALYTICAL PROCEDURES

Paper Chromatography and Paper Spotting Reactions

Paper chromatography served four purposes: a) spotting for qualitative determination of the number of possible metabolites resulting from the incubation, b) screening silica gel eluates, c) preparative chromatography for resolution of compounds not separated in the initial silica gel fractionation, and d) for suggesting chemical structure on the basis of mobility (the number and type of oxygen functions in a molecule determine its polarity and thus its relative mobility.
on paper), color reactions for the various functional groups and comparison of mobility rates of known and unknown steroids, individually or in mixed chromatograms.

The following procedures and reactions were used for the detection of steroid material on paper chromatograms.

1) An alkaline solution of triphenyl tetrazolium chloride (TPTZ) is reduced by compounds possessing an \( \alpha \)-ketol side chain to yield a red color (31). The reagent is prepared by mixing a 0.2 per cent solution of TPTZ with 10 per cent NaOH in the ratio of 2:1. The strips to be developed are passed through the reagent and appearance of the characteristic color within 30 seconds indicates a positive reaction. After the maximum color has developed, the strips are rinsed in tap water, dried in air and mounted for preservation and future reference. Some steroids with a glycerol side chain may give a positive reaction with this reagent but only after being exposed to the reagent for several minutes (35).

2) Further structural information resulting from a color reaction may be obtained with 2,4-dinitrophenylhydrazine (DNPH). It can be demonstrated that \( \Delta^4 \)-3-ketones give an orange color whereas non-conjugated 3-ketones produce a yellow color. Color formation with a 20-ketone is doubtful unless significantly high concentration (200 ug. or more per cm.) are present. Even at the high concentration mentioned, the pale yellow color produced is masked by the background color.
of the reagent absorbed by the paper which is very difficult to remove even after prolonged washing of the developed strips in running tap water. The reagent consists of a solution of DNPH in 0.5N HCl in which the strip to be developed is submerged for 15-30 minutes. Once maximum color has developed the strips are washed in running tap water, dried in air and mounted.

3) The use of an alkaline solution of m-dinitrobenzene for the determination of 17-ketosteroids has long been known (231). A number of modifications of this Zimmermann reagent have been reported, but the use of a 2 per cent m-dinitrobenzene solution in conjunction with a 2.5N alcoholic KOH solution has given exceptionally good results in the development of paper chromatograms (157). The paper strips are passed through the alkali first, excess reagent being removed by blotting, and then the strips are passed through a solution of m-dinitrobenzene, blotted again and put in an oven at 70° C. for one minute. 17-Ketone groups produce a characteristic purple color. Conjugated and non-conjugated 3-ketones give a blue color which rapidly fades to a grey and 20-ketones produce a non-specific brown color.

4) The papergrams were examined under an ultraviolet lamp (Model V-41, Ultraviolet Products Co., Pasadena, Calif.) before color development. Visual observation of a darkened zone was possible when α,β-unsaturated steroids were
Quantitative Estimation of 17-Ketosteroids

A colorimetric procedure for the quantitative estimation of steroids reacting with the Zimmermann reagent as described by Holtorff and Koch was employed for analyzing the incubation extracts and various fractions (93). Dried samples (20-100 ug.) were reacted with 0.4 ml. of 1 per cent m-dinitrobenzene in 95 per cent ethanol and 0.2 ml. 5N aqueous KOH for 90 minutes at 27° C. At the end of this period the reaction solution was diluted with 5.4 ml. 60 per cent ethanol and read at 520 μ in a Coleman colorimeter. The concentration was calculated from a standard calibration curve for dehydroepiandrosterone.

Melting Point Determination

A Koefler melting point block mounted on a microscope stage was used for the determination of all melting points which are reported here as uncorrected.

Infrared Spectrometry

The infrared absorption spectra were obtained on a 12C Perkin-Elmer spectrometer employing a sodium chloride prism. Samples were studied in the solid state as a crystalline film deposited from methylene chloride-methanol solutions (.500-1.0 mg. of sample). In the case of C₁₉ compounds, spectra were recorded in carbon disulfide solution in a micro cell.
With this method adequate spectra could be obtained with as little as 50 \( \mu \text{g.} \) of steroid.

**Sulfuric Acid Chromogen Spectra**

These spectra were obtained with a Cary Recording Spectrophotometer Model 11MS in the range 220-600 \( \text{m} \mu \). Concentrated sulfuric acid (3.0 ml.) was added to the sample and allowed to stand at room temperature for 2 hours at which time the spectra were recorded (226).

**Ultraviolet Absorption Spectra**

The ultraviolet spectra were obtained with the same instrument mentioned above. Spectra of samples in methanol (3.0 ml.) were obtained in the region from 220-350 \( \text{m} \mu \). (228).

**FORMATION OF DERIVATIVES**

**Acetylation of Steroids and Hydrolysis of Steroid Acetates**

Compounds to be acetylated were dissolved in a minimum volume of pyridine and reacted with an equal volume of acetic anhydride at room temperature overnight. The reaction mixture was diluted with 10 volumes of water and extracted with ether. The ether was successively washed with 2N HCl, 1N NaOH and distilled water until neutral. The washings were back-extracted with ether and the combined ether extracts dried over sodium sulfate, decanted and taken to dryness in vacuo.

Hydrolysis of acetates was accomplished by solution of the material in a minimum volume of methanol and an equi-
molar quantity of aqueous potassium carbonate was added. After six hours at room temperature in an atmosphere of nitrogen, acetic acid was used to neutralize the reaction mixture which was concentrated in vacuo. The concentrate was diluted with an excess of water and extracted three times with equal volumes of chloroform. The chloroform extract was washed twice with small amounts of water and dried over sodium sulfate. In those cases where difficulty of hydrolysis was encountered, sodium hydroxide was substituted for the potassium carbonate.

**Sodium Bismuthate Oxidation**

This method, developed by Brooks and Norymberski (22) permitted oxidation of either glycerol or dihydroxy acetone side chains to the corresponding 17-ketosteroid in excellent yields (approximately 70 per cent).

The steroid (10 mg.) to be oxidized was dissolved in 1.0 ml. of glacial acetic acid. To the acetic acid solution 1.0 ml. distilled water was added followed by 50 mg. of sodium bismuthate. The reaction mixture was shaken for 1/2 hour at room temperature and then diluted with distilled water and thoroughly extracted with ether. The ether extract was washed once with 2N NaOH followed by distilled water until the washings were neutral. The ether extract was dried over Na₂SO₄, decanted and concentrated to dryness.
CHAPTER VI

EXPERIMENTAL
The experimental section has been divided into two parts. Part I is concerned with the completion of an in vitro study of which preliminary results have already been reported (70). An attempt is made to develop a stepwise scheme for the in vitro transformation of a typical adrenocortical steroid by a rat liver preparation. Part II of the experimental involves a preliminary study of the fractionation of rat liver homogenate through differential centrifugation. The purpose of the fractionation is to localize individual enzyme systems in the various homogenate fractions which carry out single metabolic transformations.

PART I - INCUBATION OF 885 MG. OF 11-DEOXYCORTISOL

Rat liver (1200 g.) was homogenized in 1200 ml. of the buffered medium from which 1450 ml. of supernatant were obtained after centrifugation at 6000 x g. To each flask 10 mg. of steroid, 20 ml. of supernatant, and a DPN solution were added and the mixture incubated for two hours at 38° C. After extraction, defatting and drying, 1.98 g. of a crude extract were obtained. The extract was adsorbed on a 120 g. column of silica gel and eluted in 100 ml. volumes, a total of 78 fractions being collected as follows:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Solvent Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>Benzene</td>
</tr>
<tr>
<td>6-9</td>
<td>Benzene-ethyl acetate 19:1</td>
</tr>
<tr>
<td>10-13</td>
<td>&quot; &quot; &quot; 9:1</td>
</tr>
</tbody>
</table>
Aliquots (corresponding to 200 μg. dry weight) of the above silica gel eluates were applied to one cm. strips of paper and developed in the toluene-propylene glycol system. The results of this paper screening procedure are illustrated in Fig. 2 and the findings of the various spotting reactions are tabulated in Table I.

Fractions 26-36, after chromatography for 18 hours, yielded two major components (I and II). The slower migrating material (I) appeared to be similar to the starting material on the basis of mobility and color reactions. The second component migrating about twice as fast as I, gave a positive TPTZ, a yellow DNPH and was not detected under the ultraviolet light. Its migration rate was comparable to that of a reduced ring A derivative of the substrate (17α,21-dihydroxypregnane-3,20-dione).
Fig. 15. Results from paper chromatographic screening of silica gel fractions
Table II

Reactions on Paper of the Various Zones
Illustrated in Figure 15

<table>
<thead>
<tr>
<th>Zone</th>
<th>TPTZ</th>
<th>DNPH</th>
<th>Zimmermann</th>
<th>UV Lamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>orange</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>yellow</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>orange</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>VI</td>
<td>+</td>
<td>faint orange</td>
<td>faint</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>faint</td>
<td>orange</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>VIII</td>
<td>-</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IX</td>
<td>-</td>
<td>orange</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Fractions 35-42 were resolved individually and three distinct zones (III, IV and V) were found after chromatography for 44 hours. The fastest moving material corresponded to 11-deoxycortisol and the other two zones which migrated very closely together, gave a positive TPTZ, a negative DNPH reaction, and were not detectable under ultraviolet light. The same results were obtained with fractions 43-50.

Fractions 51-62 yielded two components (VI and VII) after a 48-hour migration period. One component was extremely polar and migrated approximately one cm. from the origin. It gave a positive TPTZ reaction and a pale yellow DNPH reaction and was not detected under the UV lamp. The second, faster migrating material gave a negative TPTZ reaction and a yellow-orange DNPH and was detected as a dark band under ultraviolet light.

The combined weights of fractions 26-62 (625 mg.) accounted for approximately 30 per cent of the weight of the extract initially adsorbed on the silica gel column. Fractions 1-25 and those beyond 63 failed to evoke positive spotting reactions with steroid reagents and were therefore not investigated further. The fractions containing steroid material were pooled for preparative paper chromatography as follows:

Pool A fractions 26-33
Pool B " 34-53
Pool C " 54-62
Pool A

Pool A (127 mg.) when applied to three 17 cm. wide strips of paper and developed in the toluene-propylene glycol system for 18 hours, yielded two components, I and II. Both zones were eluted from the paper, concentrated, and thoroughly dried in high vacuum.

Identification of Component I as 11-Deoxycortisol: The initial chromatography data suggested that this may be unreacted starting material, since it had the same mobility rate and color reactions on paper as that of 11-deoxycortisol. Crystallization from absolute ethanol yielded 20 mg. of colorless plates, m.p. 210-214°. Infrared spectrometry afforded the following information concerning the functional groups in the molecule: hydroxyl vibrations at 3495 cm.\(^{-1}\) due to C-17 and C-21 hydroxyl groups (71), a strong band at 1666 cm.\(^{-1}\) assigned to a conjugated C-3-ketone and a band near 1613 cm.\(^{-1}\) assigned to a conjugated carbonyl system confirmed the presence of \(\alpha,\beta\)-unsaturation (100) while another carbonyl absorption band near 1710 cm.\(^{-1}\) was associated with a non-conjugated hexacyclic or side chain (C-20) carbonyl groups (100). Finally, superimposition of the spectra of the unknown I and an authentic sample of 11-deoxycortisol showed that the two were identical (Fig. 16). Recovery of starting material from Pool A was approximately 3 per cent.
Fig. 16. Infrared absorption spectra of (a) authentic and (b) isolated samples of 11-deoxycortisol
Identification of Component II as 17α,21-Dihydroxyallo-
pregnane-3,20-dione: Paper chromatographic analysis offered
evidence that component II contained the α-ketol side chain
(positive TPTZ), the presence of an unconjugated C-3 carbonyl
and a reduced ring A structure (yellow DNPH and not visible
under ultraviolet light). The decreased polarity of the com-
pound on paper (faster running rate than the substrate) would
be consistent with a ring A reduced steroid. Crystallization
of the unknown from absolute ethanol yielded 50 mg. of micro-
crystalline material with no regular form melting at 204-207° C.

The material (one mg.) was deposited on a rock salt
plate and subjected to infrared analysis. A strong band near
3509 cm.⁻¹ was assigned to hydroxyl vibration of both C-17
and C-21 hydroxyl groups (71), whereas a major carbonyl ab-
sorption band near 1701 cm.⁻¹ indicated the presence of either
a hexacyclic non-conjugated (C-3) or side chain (C-20) carbonyl
or both (100). The absence of any bands near 1666 and 1613 cm.⁻¹
confirmed the reduction of ring A. A band near 1203 and 1000 cm.⁻¹
suggested a C-3 carbonyl associated with a 5α hydrogen (162).
Comparison of the spectrum of II with that of an authentic
sample of 17α,21-dihydroxyprogrenane-3,20-dione showed certain
similarities between the two compounds but sufficient differ-
ences to exclude identity. Although the infrared data sugges-
ted an allo structure, it wasn't until an authentic sample of
17α,21-dihydroxyallopregnane-3,20-dione became available that
unequivocal proof was obtained that component II was 17α,21-
dihydroxyallopregnane-3,20-dione (Fig. 17). The yield of II
was 8 per cent of the material recovered.

Pool B

Pool B (321 mg.) was applied to six 17 cm. wide
strips of paper and run for 72 hours in the toluene-propylene
glycol system. During this length of time, component V had
migrated off the paper and was collected in the run-off beaker.
Components III and IV remained on the paper.

Identification of Component V as 11-Deoxycortisol: It
was suspected that component V was unreacted substrate. It
was concentrated and dried in high vacuum and crystallization
from absolute ethanol yielded 120 mg. of colorless plates
(m.p. 212-214°C.). Superimposition of the infrared spectrum
of V with that of an authentic sample of 11-deoxycortisol (Fig.
16) proved that they were identical. The recovery of starting
material from Pool B was approximately 19 per cent.

Identification of Component III as 3α,17α,21-Trihydroxy-
allopregnan-20-one and 3β,17α,21-Trihydroxyallopregnan-20-one:
Component III was separated by means of digitonin into two
components which were identified as 3α,17α,21-trihydroxyallo-
pregnan-20-one (Fig. 18) and its 3β-hydroxy isomer (Fig. 19),
as previously described (70).
Fig. 17. Infrared absorption spectra of (a) authentic and (b) isolated samples of 17α,21-dihydroxyallopregnan-3,20-dione
Fig. 18. Infrared absorption spectra of (a) 3α,17α,21-tri-hydroxyallopregnan-20-one and (b) its diacetate
Fig. 19. Infrared absorption spectra of 3β,17α,21-trihydroxyallopregnan-20-one (a) prepared 3,21-diacetate, (b) authentic 3,21-diacetate, (c) isolated and (d) authentic samples
Identification of Component IV as 3α,17α,21-Trihydroxy-allopregnan-20-one and 3β,17α,21-Trihydroxyallopregnan-20-one: Initial infrared analysis of component IV indicated the presence of a mixture of the two compounds which had been separated in component III. Digitonin fractionation achieved resolution of the α and β compounds. The infrared spectrum of the β fraction proved to be identical with that of the 3β hydroxy component isolated from III and the infrared spectrum of the α fraction was identical to that of the 3α hydroxy component also separated from III. This appeared to be a rather paradoxical situation in which two distinct zones had been obtained with paper chromatography and yielded identical mixtures. This effect was probably due to an overloading of the paper since relatively high concentrations of 3 mg./cm. were applied to the paper. Evidently an adverse effect on the resolution of the two closely related components was experienced.

Pool C

Pool C (185 mg.) was applied to four 17 cm. wide strips of paper and chromatographed in the chloroform-formamide system for 10 hours. This permitted resolution of components VI and VII.

Identification of Component VII as 17α,20β,21-Trihydroxy-Δ⁴-pregnen-3-one: Component VII gave an orange DNPH, a faint TPTZ and was visualized as a dark band under ultraviolet light.
The mobility rate on paper indicated a structure much more polar than the tetrahydro derivatives. The faint TPTZ reaction which did not correspond to the intensity of the DNPH reaction was interpreted as originating from an \( \alpha \)-ketol contaminant (TPTZ positive). The positive UV response and the intense DNPH indicated the presence of the \( \Delta^4 \)-3-keto system. Crystallization from absolute ethanol yielded colorless needles, m.p. 187-217° C. A solid film of about 1 mg. was deposited on a rock salt plate and subjected to infrared spectrometry. A band near 3378 cm.\(^{-1}\) was assigned to hydroxyl vibration (71) while absorption bands near 1647 and 1613 cm.\(^{-1}\) established the presence of \( \alpha,\beta \)-conjugation (\( \Delta^4 \)-3-ketone) (100). A band near 1701 cm.\(^{-1}\) indicated the presence of a hexacyclic or side chain carbonyl (100). However, the intensity of the absorption band suggested that the 1701 maximum was originating from a ketonic contaminant (101). This was interpreted to mean that the conjugated and non-conjugated carbonyl groups were associated with different molecules. The intensity relationships indicated that the \( \alpha,\beta \)-unsaturated molecule could be considered the major component. Both the DNPH reaction and ultraviolet examination on the paper chromatogram fitted this infrared interpretation and permitted the conclusion that the major component was a \( \Delta^4 \)-3-ketone compound containing a glycerol side chain. Since repeated crystallizations of the material failed to resolve the mixture, it was acetylated and
the acetylated product chromatographed on paper in the ligroin-
propylene glycol system. A homogeneous zone was obtained
which gave an orange DNPH, a negative TPTZ and was visible
under ultraviolet light. Crystallization of the material
from absolute ethanol yielded colorless needles, m.p. 189-191° C.
Infrared analysis showed that an acetate had been prepared by
the presence of bands near 1239 and 1222 cm.$^{-1}$ due to the
-C-O stretching vibrations of the acetyl radical (100) and a
strong absorption near 1731 cm.$^{-1}$ characteristic of an ester
carbonyl (100). Finally, comparison of the infrared spectrum
of VII and that of an authentic sample of 17α,20β,21-trihydroxy-
Δ$^4$-pregnen-3-one-20,21-diacetate (m.p. 190-191° C.) proved to
be identical (Fig. 20). The yield of component VII as the
diacetate was approximately 4 per cent.

Identification of Component in Mother Liquor of VII as
3β,17α,20β,21-Allopregnanetetrol: The mother liquor from VII
was rerun in the toluene-propylene glycol system for 72 hours.
One zone was obtained which responded in an identical manner
as VII to the paper detection procedures. An additional color
reagent was introduced at this point in order to attempt the
detection of any non-ketonic steroid components that might be
present. The reagent used, which was developed by Rosenkrantz
for the detection of non-ketonic steroids (158) consisted of
a saturated solution of antimony trichloride in nitrobenzene.
A sample strip from the above chromatogram was passed through
Fig. 20. Infrared absorption spectra of (a) authentic and (b) isolated samples of 17α,20β,21-trihydroxy-Δ⁴-pregnen-3-one-20,21-diacetate
the reagent and heated in an oven at 90\° C. for 15 minutes. This procedure resulted in the development of a second slightly faster migrating zone. This suggested the possible presence of a ring A saturated non-ketonic steroid compound since the zone did not respond to either DNPH, TPTZ or the ultraviolet lamp. The zone was eluted, the eluate dried and crystallized from absolute ethanol. Two mg. of needle-like crystals melting between 194-197\° C. were obtained. Infrared analysis of the compound confirmed its non-ketonic nature since no carbonyl bands were present in the spectrum. Comparison of its spectrum with that of an authentic sample of 3\β,17\alpha,20\β,21-allo-pregnanetetrol (m.p. 198-200\° C.) established the identity of the unknown with the authentic sample (Fig. 21).

Characterization of Component VI: Component VI gave a positive TPTZ, a yellow DNPH reaction on paper, and a weak band under ultraviolet light. These facts suggested a reduced structure with an α-ketol side chain with possibly some unsaturated component present as a contaminant. Crystallization of the material from absolute ethanol afforded long, colorless needles, m.p. 217-222\° C. Infrared analysis indicated a reduced structure as the major component since the bands arising from a conjugated 3-ketone were of weak intensity. An intense hexacyclic or side chain type carbonyl absorption occurred. The very polar nature of the compound (10 times that of the tetrahydro compound and two times that of the unsaturated
Fig. 21. Infrared absorption spectra of (a) authentic and (b) isolated samples of 3β,17α,20β,21-allopregnane-tetrol.
component VII) was suggestive of a reduced compound with five oxygen functions in the molecule. Work is still in progress on the characterization of this component.

Run Off From Pool C

Identification of 17α,20α,21-Trihydroxy-Δ⁴-pregnen-3-one:
The run off fraction was thoroughly dried for 24 hours in high vacuum to a semi-crystalline solid. Crystallization 3 times from absolute ethanol yielded a crop of small crystals (m.p. 224-228° C.). Spotting reactions with the crystalline material on paper gave an orange DNPH, a dark band under the ultraviolet lamp and a negative reaction with TPTZ. Infrared analysis showed a strong doublet at 3356 cm.⁻¹ due to hydroxyl vibrations (71) and an intense band near 1650 cm.⁻¹ assigned to a conjugated C-3 ketone (100). A third major band near 1610 cm.⁻¹, assigned to a conjugated carbonyl system, confirmed the presence of an α,β-unsaturation (100). No bands were indicated for the presence of hexacyclic or side chain (C-20) carbonyl groups. The infrared spectroscopic data and the paper detection procedures suggested a ring A unsaturated material with a glycerol side chain. Comparison was made with the infrared spectrum of VII and although the two were quite similar, sufficient differences existed to indicate that they were not the same compounds. It was thought that this unknown might be the 20α epimer of VII. Just about this time Hübener and Schmidt-Thomeé (97) reported on the isolation of the 20α hydroxy
derivative of 11-deoxycortisol and had also published the infrared spectrum for the isolated compound. A band for band comparison of the unknown (Fig. 22) and published spectra demonstrated that the two compounds were identical.

Run Off From Pool A

The run off from pool A was chromatographed in the ligroin-propylene glycol system for 24 hours. Three zones, VIII, IX and X, were resolved.

Partial Characterization of Component X as Androstane-3,17-dione: Component X gave a purple Zimmermann, a yellow DNPH and was not visible under ultraviolet light. These properties were similar to those of androstane-3,17-dione. Since the small amount of material could not be crystallized, the material was used for infrared analysis in carbon disulfide solution. No bands were present in the hydroxyl region of the spectrum. A strong band near 1732 cm.\(^{-1}\) was assigned to the pentacyclic carbonyl vibrations of the 17-ketone (100,159) and a band near 1719 cm.\(^{-1}\) arising from the vibrations of a hexacyclic carbonyl could be referred to position 3 in the absence of hydroxyl vibrations (100). Comparison of its spectrum with that of an authentic sample of androstane-3,17-dione indicated that there were strong similarities between the two spectra but lack of definition in some of the bands of the unknown spectrum prevented unequivocal identification. The evidence
Fig. 22. Infrared absorption spectrum of isolated 17a,20α,21-trihydroxy-Δ4-pregnen-3-one
did permit a presumptive identification of X as being andro-
stane-3,17-dione.

Identification of Component IX as Δ⁴-Androstene-3,17-
dione: Component IX gave a purple Zimmermann, an orange DNPH, and was visible under ultraviolet light. Paper analysis suggested a 17-ketosteroid with an α,β-unsaturation and most likely two oxygen functions. The material was eluted from the paper, concentrated and dried thoroughly and analyzed in the infrared in carbon disulfide solution. The 17-ketosteroid nature of the compound was apparent from the strong absorption near 1732 cm.⁻¹ (159) and the evidence for the α,β-unsaturation (Δ⁴-3-keto) was afforded by the bands near 1666 and 1618 cm.⁻¹ (100). Finally, superimposition of its spectrum with that of an authentic sample of Δ⁴-androstene-3,17-dione established the identity of IX. Further evidence for identity of IX was obtained from sulfuric acid chromogen spectra and comparison of its mobility rate on paper with authentic Δ⁴-androstene-
3,17-dione.

Identification of Component VIII as Androsterone: Com-
ponent VIII gave a positive purple Zimmermann, a negative DNPH, and was not visible in ultraviolet light. The material was eluted from the paper, concentrated, thoroughly dried, and analyzed in the infrared in carbon disulfide solution. The presence of a hydroxyl group in the molecule was evidenced
by absorption near 3520 cm\(^{-1}\) and the vibrations of a penta-
cyclic carbonyl band near 1730 cm\(^{-1}\) due to the C-17 carbonyl
(100,159) was also present. Correlation of the infrared data
and the paper chromatographic analysis indicated a structure
such as androsterone. Finally, comparison of the infrared and
sulfuric acid chromogen spectra of the unknown with those of
an authentic sample of androsterone showed that the two com-
pounds were identical. The yields of components VII, IX and
X were of the order of 0.1 per cent.

PART II - INCUBATION OF 11-DEOXYCORTISOL WITH VARIOUS RAT
LIVER HOMOGENATE FRACTIONS

It is to be noted that the reduced metabolites,
isolated and identified after incubation of 11-deoxycortisol
with the rat liver preparation described in the previous sec-
tion, were all of the 5\(\alpha\) type (allopregnane or androstane).
These findings are in agreement with other workers who in-
cubated adrenocortical steroids with rat liver slices and
homogenates or perfused these compounds through isolated
whole livers (38,37,184,200).

It was generally thought that reduction exclusively
to the 5\(\alpha\) products was a species characteristic of the rat.
Recently, Tompkins and Isselbacher were able to prepare a rat
liver preparation capable of reducing cortisone to the preg-
nane (5\(\beta\)) derivative (203). They initially accomplished this
by centrifuging a rat liver homogenate at 78,000 \(x\) g., the 5\(\beta\)
reducing activity being associated with the particulate free supernatant fluid. They were not able to isolate any metabolites with the \(5alpha\) configuration (allopregnane or androstane compounds).

The only apparent difference between the tissue preparation used by Tompkins and Isselbacher and that used in Part I of this work was that in the former a complete separation of the soluble tissue components from tissue particulates had been affected. It was reasonable to assume that in the relatively more intact tissue preparations, there existed some form of interaction between the \(5alpha\) and \(5beta\) reducing enzyme systems favoring the \(5alpha\) type of activity. High speed centrifugation of the 6000 x g. supernatant should resolve these two types of activity and that the \(5beta\) should be found in the particulate-free supernatant, as has been demonstrated, and the \(5alpha\) activity should be associated with the tissue particulates.

In the following experiments only reduced components, as determined by paper chromatographic criteria, were investigated in order to determine the steric course of the reduction at \(C_5\) in the various homogenate fractions.

**Experiment A**

Rat livers (497 g.) were homogenized with 285 ml. of Krebs-Ringer phosphate buffer for 30 seconds at \(5^0\) C. in a Waring blender. 735 ml. of homogenate were obtained after
straining through several thicknesses of gauze. Centrifugation at 6000 x g. for 30 minutes yielded 250 ml. of supernatant fluid. The yield of supernatant fluid was low due to the high ratio of tissue to buffer (1 g. tissue/0.6 ml. buffer). The supernatant fluid was further centrifuged at 30,000 r.p.m. (78,000 x g.) for 90 minutes resulting in a three phase separation of the 6000 x g. supernatant fluid. Uppermost was a pink clear supernatant (100 ml.), the middle layer consisted of a fluffy particulate zone (40 ml.) and at the bottom of the tube was a layer of more closely packed particles (88 ml.). The latter two fractions were reconstituted to 100 ml. with buffer and three separate incubation experiments were run as follows:

1. Supernatant fluid (100 ml.) after 78,000 x g. centrifugation
   50 mg. of 11-deoxycortisol
   0.03 M. of nicotinamide
   0.002 M. neutralized DPN
   0.02 M. potassium hexosediphosphate
   Incubation time 2 hours at 37° C. in open flasks.

2. 100 ml. middle layer with same additions and conditions as in 1.

3. 100 ml. of the residue with same additions and conditions as in 1.
Each of the incubates were extracted separately as described in the previous section and the respective extracts were fractionated by means of preparative paper chromatography.

**Results from Incubation 1:** The extract weighing 158 mg. was applied to four 15 cm. wide strips of paper and developed in the toluene-propylene glycol system for 72 hours, a run off fraction being collected. The tetrahydro zone was eluted from the paper, concentrated and thoroughly dried in vacuo. Crystallization of the dried eluate from absolute ethanol yielded about one mg. of crystals (m.p. 217-221° C.). The material was deposited as a crystalline film on a rock salt plate and analyzed in the infrared region. Analysis of the spectra established the reduced nature of the compound and comparison was made with the spectra of each of the four tetrahydro derivatives. It proved to be identical to that of 3β,17α,21-trihydroxypregnan-20-one (Fig. 23).

Further evidence for the 5β reducing activity was obtained in the run off fraction collected. This latter fraction was rechromatographed in the ligroin-propylene glycol system for 18 hours yielding one Zimmermann reacting component. The material was eluted, dried and rechromatographed on paper for further purification. The rechromatographed material was submitted directly to infrared analysis. Comparison of its spectrum with that of an authentic sample of etiocholanolone (3α-hydroxyetiocholan-3-one) showed very strong similarities,
Fig. 23. Infrared absorption spectra of (a) authentic and (b) isolated samples of 3β,17α,21-tri-hydroxy pregnan-20-one.
especially in the fingerprint region of the spectrum where etiocholanolone gives a very characteristic triplet. Analysis of the carbonyl region of the spectrum showed in addition to a pentacyclic carbonyl (C-17 carbonyl), an absorption band due to the presence of a hexacyclic carbonyl. This suggested a possible mixture of etiocholanolone with a 3-keto,17-hydroxy derivative which would be very difficult to resolve. An experiment is suggested and which remains to be done, and that is, oxidation of the hydroxyl functions in both components to the corresponding ketones. Assuming that both compounds have the same configuration at C-5 (β), the oxidation product should be etiocholane-3,17-dione.

Results from Incubation 2: The extract (180 mg.) was applied to four 15 cm. wide strips of paper and developed in the same manner as in incubation 1. Upon color development of sample strips, two zones corresponding to tetrahydro derivatives were obtained. These were eluted as one zone and rechromatographed on paper for 72 hours. Spotting reaction once more produced the same two zones which were now eluted separately, concentrated and thoroughly dried and subjected to infrared analysis. Both components were far too impure to attempt any interpretation of the spectra and so little material was available that attempts at further purification resulted in complete loss of the material.

The run off fraction was rechromatographed in the
ligroin-propylene glycol system for 10 hours. A reduced Zimmermann positive zone was located which had the mobility rate of a saturated di-keto C₁₉ compound. The infrared spectrum of the dried eluate from this zone was found to have very strong similarities to that of an authentic sample of androstane-3,17-dione but some of the bands in the spectrum of the unknown were sufficiently obscured as to prevent unequivocal identification. An aliquot of the sample was reacted with concentrated sulfuric acid and the sulfuric acid chromogen absorption spectra recorded. The spectrum was identical to that of an authentic sample of androstane-3,17-dione. The remainder of the sample was rechromatographed on paper in admixture with an authentic sample of androstane-3,17-dione and on color development of the paper chromatogram, it was found that the mixture had run as a homogeneous zone. Correlation of the infrared and sulfuric acid chromogen spectra and the paper chromatographic analysis was considered adequate criteria to establish the identity of the unknown as that of androstane-3,17-dione. This, therefore, afforded evidence for the 5α reducing type of activity in the middle layer of the tissue fraction.

**Results from Incubation 3:** The extract (175 mg.) was applied to four 15 cm. wide strips of paper and chromatographed for 72 hours in the toluene-propylene glycol system with a run off fraction being collected. Spotting reactions produced
two tetrahydro zones. These were eluted and concentrated and rechromatographed on paper in the same manner as above. Two zones were again obtained which were eluted separately, concentrated and dried. Infrared analysis of the faster migrating zone and comparison of its spectrum with that of the previously isolated 3α,17α,21-trihydroxyallopregnan-20-one proved that the two were identical. The slower migrating tetrahydro zone yielded too little material for identification.

Experiment B

In this latter experiment it was decided to use a more dilute homogenate in order to effect better packing of tissue particulates.

500 g. of rat liver were homogenized with 500 ml. of buffer. After straining through gauze, 880 ml. of homogenate were obtained, which, after centrifugation at 6000 x g. for 1/2 hour, were separated into 475 ml. of supernatant and 380 ml. of residue. 400 ml. of the supernatant were centrifuged at 30,000 r.p.m. (78,000 x g.) for 90 minutes. In this case the tissue particulates were packed into one zone. Three separate incubations were run as follows:

   170 mg. of deoxycortisol
   0.03 M nicotinamide
   0.002 M DPN
   0.02 M potassium hexose diphosphate
   Incubation time 2 hours at 37° C.
5. Residue after centrifugation at 78,000 x g. reconstituted to 340 ml. with buffer.
   Same conditions and additions as in 4.

6. Residue from 6000 x g. centrifugation reconstituted to 880 ml. with buffer.
   Same additions and conditions as in 4 were employed, except that only 120 ml. of tissue preparation and 60 mg. of 11-deoxycortisol were incubated.

The individual incubations were extracted as previously described.

**Results from Incubation 4:** The extract (290 mg.) was chromatographed on eight 15 cm. wide strips of paper in the toluene-propylene glycol system for 72 hours and the run off fraction was collected. The zone corresponding to the tetrahydro derivative was eluted, concentrated, and dried. Crystallization from absolute ethanol yielded an initial crop of 11 mg. of microcrystals with no definable form melting between 206-210° C. The infrared spectrum of this compound proved to be identical to that of an authentic sample of 3α,17α,21-trihydroxy-pregn-20-one (Fig. 24). This latter compound differed from the tetrahydro derivative isolated in Experiment A, Incubation 1 in the configuration of the hydroxyl at C-3, although they were both pregnane (5β) derivatives.
Fig. 24. Infrared absorption spectra of (a) authentic and (b) isolated samples of 3α,17α,21-trihydroxy-pregn-20-one
Results from Incubation 5: An aliquot of the extract (1/10 of 392 mg.) was applied to a 15 cm. wide strip of paper and chromatographed in the toluene-propylene glycol system for 72 hours with a run off fraction being collected. Spotting reactions on paper failed to locate any zones wholly characteristic of tetrahydro derivatives. All zones gave reactions typical of compounds with a $\Delta^4$-3-ketone system in the molecule, in addition to a positive TPTZ. One zone corresponding to that of a tetrahydro derivative in polarity was eluted and analyzed in the infrared. Band analysis of the carbonyl region suggested that one of the major components possessed an $\alpha,\beta$-unsaturation. In addition there was a carbonyl present near the region normally assigned to pentacyclic carbonyl vibrations and also a carbonyl absorption characteristic of hexacyclic and side chain carbonyls. The carbonyls seemed to belong to different molecules since they differed in relative intensities from each other. An aliquot of the paper eluate was subjected to ultraviolet analysis and a sharp band was obtained at 240 $\mu$m. It was estimated from this that the $\alpha,\beta$-unsaturated component accounted for 40-50 per cent of the total weight of the dried eluate. Quantitative Zimmermann determination on another aliquot gave a value of 20 per cent 17-ketosteroid material present in the mixture. The third component in the mixture could either be a reduced steroid or some non-steroidal contaminant.
A 5 mg. sample of this mixture was oxidized with sodium bismuthate in order to reduce the oxidizable components in the mixture to the simpler C₁₉ compounds.

The oxidized product (approximately 3.5 mg.) was chromatographed in the ligroin-propylene glycol system for 18 hours. Color development with the Zimmermann reagent showed the presence of four zones, two major and two minor zones. Of the two major zones, one appeared to be a reduced C₁₉-17-ketosteroid, of which the paper chromatographic behavior could fit either androsterone or epiandrosterone. Infrared analysis of the material and comparison of its spectrum with that of an authentic sample of isoandrosterone proved that they were identical. Identification of this degradation product afforded evidence for the 5α reducing type of activity in this tissue fraction.

**Results from Incubation 6:** The extract weighing 103 mg. was applied to three 15 cm. wide strips of paper and developed in the toluene-propylene glycol system for 72 hours, a run off fraction being collected. Two zones corresponding to tetrahydro derivatives were developed. These were eluted separately and rechromatographed in the toluene-propylene glycol system for 72 hours. Each component, after elution from paper and drying, was crystallized from absolute ethanol. Infrared analysis of the two components proved that the spectrum of one was identical to that of 3α,17α,21-trihydroxyallo-
pregnan-3-one and the spectrum of the other identical to that of 3β,17α,21-trihydroxyallopregnan-20-one.

**Isolation and Identification of 6β-Hydroxy-11-deoxycortisol**

The remainder of the extract (350 mg.) from incubation 5, Experiment B was adsorbed on a 25 g. silica gel column and chromatographed. A series of seventy-five 25 ml. fractions were collected. An aliquot of each fraction (200 ug.) was chromatographed in the toluene-propylene glycol system for screening purposes. Of immediate interest were a group of polar fractions which had been eluted from the silica gel column with ethyl acetate. Each fraction possessed one common paper chromatographic zone which gave an intense TPTZ reaction, an orange DNPH and was visible under the ultraviolet lamp.

These fractions were pooled (total weight 37 mg.) and applied on one 15 cm. wide strip of paper and chromatographed for 80 hours in the toluene-propylene glycol system. Color development with TPTZ and DNPH disclosed four zones (Table III). The two faster migrating zones (a and b) behaved like ring A unsaturated compounds possessing a glycerol side chain since they gave an orange DNPH, were visible under the ultraviolet lamp but were negative to TPTZ. The two more polar zones (c and d) gave a positive TPTZ, an orange DNPH and were visible under the ultraviolet lamp. All four zones were eluted and attention was centered on the two more polar zones (c and d) since their behaviour suggested possible hydroxylated products.
Fig. 25. Infrared absorption spectra of (a) authentic and (b) isolated samples of 6β,17α,21-trihydroxy-Δ4-pregnen-3,20-dione
Table III
Results After Chromatographing Pool E in Toluene-propylene Glycol System for 80 Hours

<table>
<thead>
<tr>
<th>Zone</th>
<th>TPTZ</th>
<th>DNPH</th>
<th>Ultraviolet lamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>-</td>
<td>orange</td>
<td>+</td>
</tr>
<tr>
<td>b</td>
<td>-</td>
<td>orange</td>
<td>+</td>
</tr>
<tr>
<td>c</td>
<td>+</td>
<td>orange</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
<td>+</td>
<td>orange</td>
<td>+</td>
</tr>
</tbody>
</table>
Component d was crystallized from absolute ethanol, yielding a 4 mg. crop of colorless plates melting between 240-245° C. Infrared analysis of the compound and comparison of its spectrum with that of an authentic sample of 6β-hydroxy-11-deoxycortisol (m.p. 243-246° C.) proved that the two were identical (Fig. 25).

Component d (11.0 mg.) was similarly crystallized from absolute ethanol and yielded 6 mg. of powdery material which melted between 198-218° C. Infrared analysis indicated a mixture of ring A saturated and unsaturated components. Recrystallization did not improve the melting point nor the infrared spectrum. Work is still in progress on the resolution of the mixture and identification of the components.
CHAPTER VII

DISCUSSION AND CONCLUSIONS
REDUCTION OF THE RING A UNSATURATION

When a comparison was made between the \textit{in vivo} and \textit{in vitro} metabolism of the C\textsubscript{21} adrenocortical steroid, certain marked differences were apparent. After administration of C\textsubscript{21} adrenocortical steroid hormones to human subjects, the \textit{in vivo} reduction of ring A yielded predominantly 5\beta (C\textsubscript{21} pregnane or C\textsubscript{19} etiocholane) products in the urine. This applied as well to the \textit{in vivo} metabolism of progesterone in human subjects and in the rabbit. However, \textit{in vitro} studies with C\textsubscript{21} corticoids and rat liver tissue indicated that the reduction of the $\Delta^4$ group proceeded exclusively to the 5\alpha (C\textsubscript{21} allopregnane or C\textsubscript{19} androstane isomers. This was the case after the perfusion of cortisone (37,38) and cortisol (36) through the intact liver, incubation of deoxycorticosterone with rat liver slices (184) and the incubation of progesterone with rat liver homogenate (200). It was tempting to explain this difference on the basis of a species difference since most \textit{in vivo} studies were done with human subjects whereas the \textit{in vitro} studies have utilized rat liver tissue. However, species differences did not correspond to the available facts since Horwitt and Segaloff (96) incubated progesterone with rabbit liver slices and isolated pregnane-3\alpha,20\alpha-diol and Taylor (201) incubated progesterone with rabbit liver homogenates and
isolated both allopregnane (5α) and pregnane (5β) compounds. Furthermore, Tomkins and Isselbacher (203) incubated cortisone with a highly purified rat liver homogenate supernatant preparation and showed conversion to the pregnane (5β) derivative exclusively.

In this research, incubation of 11-deoxycortisol with a 6000 × g supernatant from rat liver homogenate resulted in the isolation and identification of five ring A reduced conversion products (Table IV). The configuration at C-5 in each compound was 5α which was similar to the findings of Caspi et al. (38), Schneider (184) and Taylor (200). However, these observations are in sharp contrast to the results of Tomkins and Isselbacher (203) who observed ring A reduced metabolites of the 5β series. The difference between the tissue preparations employed in the two investigations was in the centrifugal force applied to the initial liver homogenates. Tomkins and Isselbacher centrifuged the homogenate at 78,000 × g to obtain a particulate free supernatant whereas in this work the centrifugation of the homogenate was carried out at 6000 × g to yield a supernatant not completely free of particulate matter. It was felt that the supernatant employed in our studies probably contained both the 5α and the 5β reducing enzymes and that for some unexplained reason only
Table IV

Conversion Products Isolated After the Incubation of 11-Deoxycortisol with the Various Rat Liver Homogenate Fractions

<table>
<thead>
<tr>
<th>6000 x g Supernatant</th>
<th>6000 x g Residue</th>
<th>78,000 x g Residue</th>
<th>Particulate free Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α,21-Dihydroxyallo-</td>
<td>3α,17α,21-trihydroxy-allopregnane-3,20-dione</td>
<td>3α,17α,21-trihydroxy-allopregnan-20-one</td>
<td>3α,17α,21-trihydroxy-allopregnan-20-one</td>
</tr>
<tr>
<td>pregnane-3,20-dione</td>
<td>3α,17α,21-trihydroxy-</td>
<td>3β,17α,21-trihydroxy-</td>
<td>3β,17α,21-trihydroxy-</td>
</tr>
<tr>
<td></td>
<td>allopregnan-20-one</td>
<td>allopregnan-20-one</td>
<td>allopregnan-20-one</td>
</tr>
<tr>
<td>3α,17α,21-trihydroxy-</td>
<td>3β,17α,21-trihydroxy-</td>
<td>Androstane-3,17-dione</td>
<td>Etiocholanolone</td>
</tr>
<tr>
<td>allopregnan-20-one</td>
<td>allopregnan-20-one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α,20β,21-trihydroxy-</td>
<td>Epiandrosterone*</td>
<td>6β,17α,21-trihydroxy-</td>
<td></td>
</tr>
<tr>
<td>Δ^4^-pregnen-3-one</td>
<td></td>
<td>Δ^4^-pregnen-3,20-dione</td>
<td></td>
</tr>
<tr>
<td>17α,20α,21-trihydroxy-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ^4^-pregnen-3-one</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3β,17α,20β,21-allo-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pregnanetetrol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ^4^-androstene-3,17-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dione</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>androsterone</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Product of Sodium Bismuthate oxidation
the 5α steroids could be realized. However, since Tomkins and Isselbacher observed 5β reduction, it was further felt that if our supernatant was subjected to 78,000 x g the new supernatant might contain the 5β reducing enzyme while the new residue might contain the 5α reducing enzyme. Accordingly, a new series of experiments were undertaken in which the rat liver homogenate was subjected to differential centrifugation. The liver homogenate was centrifuged first at 6000 x g to obtain a residue and a supernatant and the supernatant further centrifuged at 78,000 x g to obtain a second residue and a particulate free supernatant. Incubation of 11-deoxycortisol with the 78,000 x g supernatant and the two residue fractions was carried out with the prime objective being to isolate and identify only ring A reduced conversion products in order to study the steric course of the ring A reduction in the various homogenate fractions. The 78,000 x g supernatant yielded reduced metabolites exclusively of the 5β series, whereas the particulate fractions yielded metabolites exclusively of the 5α form (Table IV). This is the first reported instance demonstrating the presence and separation of both the 5α and 5β reducing enzymes in rat liver tissue. In view of the findings of this investigation and those of Taylor with rabbit liver homogenate (201), consideration of a species difference between human and animals with respect to the steric course of the reduction at C-5 becomes less likely. However quantitative re-
relationships still remain to be established. The results just reported for this investigation pose an obvious question. Why is it when C$_{21}$ adrenocortical steroids are perfused through the intact rat liver, or incubated with rat liver slices, homogenate and a 6000 x g supernatant from a rat liver homogenate, that the reduction proceeds exclusively to the 5α form? Yet separation of the particulate matter from the soluble tissue components by centrifugation at 78,000 x g makes the 5β reducing activity become apparent in the soluble fraction. A possible explanation may be that the phenomenon may be due to the presence of an inhibitor of the 5β reducing enzyme system which is separated from the soluble fraction by centrifugation at 78,000 x g. Such a possibility has suggested a future experiment in which the particulate matter is added back to the supernatant to test for any inhibitory material on the 5β reducing enzyme system. The second possibility to consider is that one enzyme system may be masking the activity of the other by virtue of its greater reactivity. Certainly, competition for substrate need not be considered, since in this work large excesses of substrate were employed.

One must also emphasize the point, that in vivo metabolism studies in the rat are indicated to parallel the in vivo human metabolism experiments. Such studies would determine whether or not both the 5α and 5β reducing enzyme systems are operative in the intact animal both from a qualitative
and quantitative point of view. Conversely, in vitro data with human liver tissue is lacking for comparison with the in vivo human data. Obviously, collection of tissue in this latter case presents a serious problem. Nevertheless, it is conceivable that experiments could be designed to work with biopsy samples and highly radioactive adrenocortical steroids and identification of the ring A reduced metabolites established by the radioisotope dilution technique. Ring A reduction in vitro of C21 steroids with various liver preparations is summarized in Table V.

REDUCTION OF THE C3-KETONE

Closely associated in sequence with ring A saturation is reduction of the C-3 keto function of the C21 adrenocortical steroids. The result being little or no accumulation of the dihydro derivative in either in vivo or in vitro metabolism experiments or at best the dihydro product is a minor one. However in this work the dihydro derivative of deoxycorticisol was one of the major conversion products isolated after the incubation of 11-deoxycortisol with a 6000 x g supernatant of a rat liver homogenate. Taylor (200) isolated the dihydro derivative of progesterone in approximately 10% yield after incubation of the latter with a rat liver homogenate in agreement with the findings of this investigation. Schneider (184) who incubated deoxycorticosterone with rat liver slices was able to isolate only minor amounts of the dihydro deriva-
Table V

Ring A reduction of C\textsubscript{21} Steroids with Various Liver Preparations In Vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue Preparation</th>
<th>Configuration at C-5</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>Rat liver perfusion</td>
<td>5(\alpha)</td>
<td>Caspi et al. (38)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Caspi (36)</td>
</tr>
<tr>
<td>Cortisone</td>
<td>78,000 x g supernatant preparation</td>
<td>5(\beta)</td>
<td>Tomkins and Isselbacher (203)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Rat liver homogenate</td>
<td>5(\alpha)</td>
<td>Taylor (200)</td>
</tr>
<tr>
<td></td>
<td>Rabbit liver homogenate</td>
<td>5(\alpha), 5(\beta)</td>
<td>&quot; (201)</td>
</tr>
<tr>
<td></td>
<td>Rabbit liver slices</td>
<td>5(\beta)</td>
<td>Horwitt and Segaloff (96)</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>Rat liver slices</td>
<td>5(\alpha)</td>
<td>Schneider (184)</td>
</tr>
<tr>
<td>11-Deoxy-cortisol</td>
<td>Rat liver supernatant 6000 x g</td>
<td>5(\alpha)</td>
<td>This investigation</td>
</tr>
<tr>
<td></td>
<td>6000 x g residue of rat liver homogenate</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>78,000 x g residue of rat liver homogenate</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>78,000 x g supernatant of rat liver homogenate</td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>
tive after the perfusion of cortisone through intact rat livers. It is difficult to explain this relatively large accumulation of dihydro derivative observed in this investigation since both Ungar and Dorfman (208) and Tomkins and Isselbacher (203) have shown that reduction of the dihydro to the tetrahydro form proceeded very readily in rat liver tissue preparations. The results may be explicable on the basis of lowered concentration of 3-keto reducing enzyme systems incurred during preparation of the tissue, or to a limiting concentration of oxidizable substrate for regenerating reduced coenzyme (DPNH) which is apparently necessary for the reduction of dihydro to the tetrahydro form. However, since the reverse reaction, oxidation of tetrahydro to the dihydro form, has been demonstrated to take place both in vivo and in vitro, it is conceivable that in this particular case conditions were such as to shift the equilibrium to the left favoring the accumulation of dihydro derivative in the system. It is apparent that in order to test these possibilities more detailed studies are needed. Co-factor requirements would need to be established in addition to the separation of the ring A reducing enzyme system from the 3-keto reducing enzyme systems which has thus far not been demonstrated for mammalian tissue. Such a separation would be highly desirable in order to study the properties of each individual system.

Reduction of the C-3 ketone function in vivo after the administration of C21 adrenocortical steroids to humans,
seemed to proceed preferentially to the 3α-hydroxy form. In in vitro studies both the 3α-hydroxy and 3β-hydroxy forms have been isolated and identified. Species considerations need not enter into this, since progesterone administered in vivo to the rabbit has been shown to be converted to the 3α-hydroxy derivative by Heard et al. (85). However, certain differences were observed between incubations of various adrenocortical steroid hormones with liver tissue preparations. Horwitt and Segaloff (96) incubated progesterone with rabbit liver slices and Taylor (200) incubated progesterone with rat liver homogenate and in each instance only the 3α isomer was isolated. But when Taylor (201) incubated progesterone with a rabbit liver homogenate he was able to isolate both the 3α and 3β-hydroxy epimers, the 3α being the major form. When Tomkins and Isselbacher (203) incubated cortisone with a highly purified supernatant preparation from rat liver homogenate only the 3α-hydroxy compound could be isolated. On the other hand, Caspi et al. (38) isolated both the 3α and 3β-hydroxy epimers from the perfusion of cortisone through rat livers. However, when cortisol was used as the substrate only the 3β-hydroxy epimer was isolated. Caspi (36) suggested that the presence of an 11β-hydroxy function on the cortisol nucleus may have influenced the steric path of the enzymatic reduction. Ungar et al. (208) incubated the dihydroprogrenane derivative of 11-deoxycortisol and demonstrated the
reduction of the C-3 keto group to proceed mainly to the 3α hydroxy form, very little 3β-hydroxy epimer being realized. In this research, incubation of 11-deoxycortisol with the 6000 x g supernatant from a rat liver homogenate demonstrated the reduction to both the 3α and 3β-hydroxy derivatives, the 3α epimer predominating over the 3β in a ratio of 2:1. Similarly, incubation with the residue fractions obtained after differential centrifugation of the rat liver homogenate and the 78,000 x g supernatant all yielded both epimers and in each case the 3α form predominated.

The possible influence of nuclear substituents on the steric course of the reduction at C-3 has been suggested. Furthermore Ungar and Dorfman (205) have carried out extensive studies with 3-keto ring A reduced C₂₁ steroids with and without substituents at positions 11 and 17, in order to determine the influence of these substituents on the steric course of the reduction at C-3. Supernatant and residue fractions from a rat liver homogenate were used. They observed that C₂₁-17-desoxy compounds (21-hydroxyallopregnane-3,20-dione and pregnane-3,20-dione) incubated with the supernatant fraction were reduced to 3β-hydroxy compounds, whereas the C₂₁-17-hydroxy compounds (17α,21-dihydroxypregnane-3,11,20-trione, 11β,17α,21-trihydroxypregnane-3,20-dione and 17α,21-dihydroxypregnane-3,20-dione) incubated with the supernatant fraction were reduced to 3α-hydroxy compounds. The C₂₁-17-hydroxy compounds, when incubated with the residue fraction still yielded 3α-
hydroxy derivatives, whereas the C\textsubscript{21} desoxy compounds incubated with the residue fraction, in contrast with results obtained with the supernatant fraction, were reduced to 3\(\alpha\)-hydroxy derivatives. In each instance the activity of the residue tissue fraction was considerably lower than that of the supernatant tissue fraction. Furthermore, the 11\(\beta\)-hydroxyl substituent did not appear to have any influence on the steric course of the reduction at C-3. The foregoing discussion has indicated considerable lack of information for a more definitive understanding of the influence of steroid structure on the steric course of the reduction at C-3. Purification and concentration of the responsible enzyme systems and demarcation of their properties would appear to be most pertinent. The steric course of the in vitro reduction of C\textsubscript{21} steroids of C-3 in various liver preparations is summarized in Table VI.

REDUCTION OF THE C-20 KETONE

Reduction of the C-20 carbonyl of the steroid hormones may also proceed to either the 20\(\alpha\) or 20\(\beta\)-hydroxy epimer. When a comparison is made between \textit{in vivo} and \textit{in vitro} results certain differences manifest themselves. Administration of adrenocortical steroids \textit{in vivo} to human subjects has resulted in the excretion in urine of the 20\(\alpha\)-hydroxy metabolites exclusively. This has also been observed for the human and the rabbit receiving progesterone. However, Burstein and Dorfman (24) after the administration of cortisol to guinea pigs, were able to isolate both the 20\(\beta\) and the 20\(\alpha\) hydroxy epimers. \textit{In vitro}, perfusion of cortisone through rat livers yielded only
Table VI

Steric Course of Reduction of C21 Steroids at C-3 in Various Liver Tissue Preparations In Vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue Preparation</th>
<th>Configuration at C-3</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>Rat liver perfusion</td>
<td>3α,3β</td>
<td>Caspi et al. (38)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&quot;</td>
<td>3β</td>
<td>Caspi (36)</td>
</tr>
<tr>
<td>Cortisone</td>
<td>Purified supernatant from rat liver homogenate</td>
<td>3α</td>
<td>Tomkins and Isselbacher (203)</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>Rat liver slices</td>
<td>3α,3β</td>
<td>Schneider (184)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Rat liver homogenate</td>
<td>3α</td>
<td>Taylor (200)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>3α,3β</td>
<td>Taylor (201)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>3α</td>
<td>Horwitt &amp; Segaloff (96)</td>
</tr>
<tr>
<td>17α,21-Dihydroxyprogland-3,20-dione</td>
<td>Rat liver supernatant and residue</td>
<td>3α,3β</td>
<td>Ungar and Dorfman (208)</td>
</tr>
<tr>
<td>Dihydrocortisol-5β</td>
<td>Rat liver supernatant and residue</td>
<td>3α</td>
<td>Ungar (205)</td>
</tr>
<tr>
<td>Dihydroprogesterone-5β</td>
<td>Rat liver supernatant and residue</td>
<td>3β,3α</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dihydro DOC-5β</td>
<td>Rat liver supernatant and residue</td>
<td>3β,3α</td>
<td>&quot;</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>6000 x g supernatant from rat liver homogenate</td>
<td>3α,3β</td>
<td>This investigation</td>
</tr>
<tr>
<td></td>
<td>6000 x g residue from rat liver homogenate</td>
<td>3α,3β</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>78,000 x g residue from rat liver homogenate</td>
<td>3α,3β</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>78,000 x g supernatant from rat liver homogenate</td>
<td>3α,3β</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

*The preponderant epimer (3α-hydroxy or 3β-hydroxy) is underscored.*
the 20β-hydroxy derivatives were obtained. The same epimer was isolated after the incubation of pregnane-3,20-dione and pregnanolone with a chick liver homogenate (207). In each instance after the incubations of progesterone with rabbit liver homogenate (201), slices (96), 11-deoxycortisol with rat liver (219) and pig liver (77) homogenates, the only reduced product isolated was the 20α hydroxy epimer. However, in this investigation, both the 20α and the 20β-hydroxy isomers were isolated and identified after the incubation of 11-deoxycortisol with the 6000 x g supernatant from a rat liver homogenate. This is the first reported instance of the isolation of both isomers from any one in vitro system.

Localization of the 20-reducing activity in the tissue particulates (78,000 x g and 6000 x g residues) was established by paper-partition chromatography criteria such as color reactions and mobility rates on paper chromatograms. Such activity could not be visualized in the particulate free supernatant. This does not preclude the absence of 20-reducing activity in this latter fraction, since completely reduced non-ketonic compounds may have been present. This group of compounds do not lend themselves readily to characterization on paper chromatograms and could be very easily missed. Steric course of the in vitro reduction of C21 steroids at C-20 in various liver tissue preparations is summarized in Table VII.
Table VII

Steric Course of the In Vitro Reduction of $C_{21}$ Steroids at C-20 in Various Liver Tissue Preparations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue Preparation</th>
<th>Configuration at C-20</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>Rat liver perfusion</td>
<td>20$\beta$</td>
<td>Caspi et al. (38)</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>Rat liver slices</td>
<td>?</td>
<td>Schneider (184)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Rabbit liver slices</td>
<td>20$\alpha$</td>
<td>Horwitt and Segaloff (96)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Rabbit liver homogenate</td>
<td>20$\alpha$</td>
<td>Taylor (201)</td>
</tr>
<tr>
<td>Pregnane-3,20-dione</td>
<td>Chick liver</td>
<td>20$\beta$</td>
<td>Ungar (207)</td>
</tr>
<tr>
<td>Pregnanolone</td>
<td>Chick liver</td>
<td>20$\beta$</td>
<td>Ungar (207)</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>Rat liver homogenate</td>
<td>20$\alpha$</td>
<td>Hübener and Schmidt-Thomé (219)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Pig liver homogenate</td>
<td>20$\alpha$</td>
<td>Hayano (77)</td>
</tr>
<tr>
<td>&quot;</td>
<td>6000 x g supernatant from rat liver homogenate</td>
<td>20$\alpha$ and 20$\beta$</td>
<td>This investigation</td>
</tr>
</tbody>
</table>
OXIDATIVE REMOVAL OF THE STEROID SIDECHAIN

It was early recognized that the administration of certain adrenocortical steroids to human subjects resulted in an increased excretion of 17-ketosteroids in urine. This has become a very commonly observed and studied phenomenon. The prerequisite for a 17-ketogenic adrenocortical steroid is the presence of the 17α-hydroxy group on the molecule since administration of compounds like deoxycorticosterone, corticosterone and progesterone did not give rise to an increased excretion of 17-ketosteroids in urine. Only two reports have appeared concerning the investigation of 17-ketosteroid formation from adrenocortical hormones in an isolated tissue preparation and in which definitive isolation and identification of the end products have been effected. Caspi et al. (38) and Caspi and Hechter (37) have isolated two 17-ketosteroids from the perfusion of cortisone through the intact rat liver. In the present investigation, it was possible to successfully isolate and identify three 17-ketosteroids and tentatively identify a fourth one (Table IV). The study now being reported, therefore, is the first instance in which liver homogenate preparations have been shown to be capable of degrading the adrenocortical steroid side chain. This, in addition, demonstrates that intact cells are not necessary for the reaction to take place and provides a means to establish the optimal conditions for studying the reaction and concentrating the enzyme system.
HYDROXYLATION AT CARBON 6

Direct evidence in vivo for the introduction of an oxygen function at carbon 6 of the steroid nucleus has been demonstrated by Burstein and Dorfman (24). After the administration of cortisol to guinea pigs, the 6β-hydroxy derivative was isolated and identified. Burstein et al. were also successful in isolating the same compound from human urine (182) and from guinea pig urine (26). Other workers (180,181) have isolated 6α-hydroxy compounds from human urine. In vitro the presence of 6-hydroxylating enzyme systems in tissue has been demonstrated for adrenal (126,75,106), corpus luteum (82), placenta (155) and certain microbiological species (131). Axelrod and Miller (3) have presented paper chromatographic evidence for the conversion of deoxycorticosterone, Δ4-androstene-3,17-dione and testosterone to their respective 6β-hydroxylated derivative after perfusion through cirrhotic rat livers and in a similar manner Miller and Axelrod (129) have shown the conversion of cortisone to 6β-hydroxydeoxycorticosterone. In this investigation, the incubation of 11-deoxycortisol with the 78,000 x g particulate fraction from rat liver homogenate has resulted in the isolation and unequivocal identification of the 6β-hydroxy derivative. Axelrod and Miller (3) and Miller and Axelrod (129) advanced the suggestion that 6-hydroxylation may be characteristic of the cirrhotic liver. This need not be the case, since in this investigation, 6β-hydroxylation has been unequivocally demonstrated in rat liver tissue ob-
tained from normal healthy animals. Since Miller and Axelrod (129) observed a depressed ability of the perfused cirrhotic liver to reduce the ring A α,β-unsaturation, an interpretation can be made which is more in keeping with the facts. Any steroid which had undergone 6-hydroxylation in a cirrhotic liver preparation would be affected in a limited manner by further reduction in ring A and would lend itself much more readily to visualization and isolation than ring A reduced analogues, especially when using paper chromatographic separation techniques. This has been the experience in this research. When incubations were carried out with in vitro systems which were highly active with regards to ring A reduction, mixtures of highly polar material were isolated suggestive, in their mobility and chemical behaviour, of ring A reduced C_{21}O_{5} compounds. These mixtures have to date evaded resolution and identification. On the other hand, when the 78,000 x g particulate fraction was used, which apparently possessed minor activity with regards to ring A reduction, sufficient quantities of 6-hydroxylated compounds, unaffected by further reduction, accumulated in the system. This made the isolation and identification of the 6β-hydroxylated compound possible with relative ease. The question of 6α-hydroxylation in liver tissue remains open. Paper chromatographic data has indicated the presence of a second component, slightly more polar than the 6β-hydroxylated derivative but reacting in an identical manner to the latter compound with respect to reactive func-
tional groups. Even though crystalline material was obtained from this fraction, infrared analysis indicated a mixture of saturated and unsaturated components. Attempts to identify the components in the mixture are still in progress.

Based on the results just reported for this investigation, a tentative scheme for the in vitro metabolism of 11-deoxycortisol (I) in rat liver tissue is proposed (Fig. 26). Reduction of ring A results in the production of the two possible dihydro derivatives, 17α,21-dihydroxy-pregnane-3,20-dione (II) and 17α,21-dihydroxyallopregnane-3,20-dione (III). Further reduction of II and III at C-3 yields the four tetrahydro isomers, 3β,17α,21-trihydroxyallopregnan-20-one (IV), 3α,17α,21-trihydroxyallopregnan-20-one (V), 3α,17α,21-trihydroxypregnan-20-one (VI) and 3β,17α,21-trihydroxypregnan-20-one (VII). The four tetrahydro isomers upon reduction at C-20 can theoretically produce eight isomers of which only 3β,17α,20β,21-allopregnanetetrol (VIII) has been isolated in this study. Reduction at C-20 can occur without reduction of ring A with the formation of the two epimers 17α,20α,21-trihydroxy-Δ⁴-pregnen-3-one (XIV) and 17α,20β,21-trihydroxy-Δ⁴-pregnen-3-one (XV). The C₁₉ derivative Δ⁴-androstene-3,17-dione (IX) can arise from oxidative degradation of I, XIV or XV and follow reductive pathways to etiocholane-3,17-dione (XI), androstane-3,17-dione (XII), etiocholanolone (X) and androsterone (XIII). In addition XI and XII can arise from side chain scission of III and IV
Fig. 26. Tentative scheme for the in vitro metabolism of 11-deoxycortisol in rat liver tissue.
and then be reduced to $X$ and $X_{III}$ respectively. Furthermore $X$ and $X_{III}$ can be derived from the respective tetrahydro derivatives $VI$ and $V$. Finally hydroxylation at carbon 6 can yield $6\beta,17\alpha,21$-trihydroxy-$\Delta_4$-pregnene-3,20-dione ($X_{VII}$) and the $6\alpha,17\alpha,21$-trihydroxy-$\Delta_4$-pregnene-3,20-dione ($X_{VI}$). Thus far only the presence of the $6\beta$-hydroxy isomer ($X_{VI}$) has been established.
CHAPTER VIII

SUMMARY
The liver has been well established as being one of the most important organs involved in the metabolism of adrenocortical steroids. Liver perfusion, incubations with liver slices, homogenates and purified liver preparations all effect extensive changes on the steroid nucleus.

In order to develop a step-wise scheme for the in vitro metabolism of a typical adrenocortical steroid and to localize enzyme systems effecting single metabolic transformations, 11-deoxycortisol was incubated with various rat liver homogenate fractions and the conversion products isolated and identified.

The liver fractions were obtained by differential centrifugation of liver homogenates. This procedure afforded a 6000 x g supernatant, a 6000 x g residue (corresponding to mitochondria in sedimentation properties), a 78,000 x g residue (corresponding to microsomes in sedimentation properties) and a 78,000 x g particulate free supernatant. Each tissue preparation was incubated with 11-deoxycortisol at 37° C. for two hours with flasks open to the atmosphere. A tissue to steroid ratio of approximately 1000:1, based on the initial weight of the liver, was employed.

After extraction of the incubation mixtures with suitable solvents, the dried extracts were further purified and resolved into individual components by silica gel adsorption and paper partition chromatography, digitonide formation and crystallization. Identifications were established by
melting points, infrared absorption spectroscopy, ultraviolet spectrophotometry in methanol and in concentrated sulfuric acid.

This made possible the identification of twelve transformation products and the tentative identification of a thirteenth. These products are listed as follows:

I 17α,21-dihydroxyallopregnane-3,20-dione
II 3α,17α,21-trihydroxyallopregnan-20-one
III 3β,17α,21-trihydroxyallopregnan-20-one
IV 3α,17α,21-trihydroxypregnan-20-one
V 3β,17α,21-trihydroxypregnan-20-one
VI 3β,17α,20β,21-allopregnanetetrol
VII 17α,20β,21-trihydroxy-Δ⁴-pregnen-3-one
VIII 17α,20α,21-trihydroxy-Δ⁴-pregnen-3-one
IX 6β,17α,21-trihydroxy-Δ⁴-pregnene-3,20-dione
X androsterone
XI androstane-3,17-dione
XII Δ⁴-androstene-3,17-dione
XIII etiocholanolone (tentatively identified)

Reduction of ring A to the 5α (allopregnane or androstane) derivatives has been demonstrated by the isolation and identification of I, II, III, X and XI whereas reduction to the 5β (pregnane or etiocholane) derivatives has been demonstrated by the isolation and identification of III, IV, and XIII. Other investigators employing either rat liver
slices, homogenates or perfusion of the isolated rat liver, were able to isolate only 5α reduced products. This was undoubtedly due to the type of tissue preparation used, since in this investigation incubation of 11-deoxycortisol with the 6000 x g supernatant, and the particulate fractions sedimenting at 6000 x g and 78,000 x g possessed exclusively the Δ^4-5α-hydrogenase activity, whereas the particulate-free supernatant obtained after centrifugation at 78,000 x g resulted exclusively in 5β products. This demonstrated the presence of only the Δ^4-5β-hydrogenase enzyme system in the particulate-free supernatant (78,000 x g). The reason for the apparent lack of the Δ^4-5β-hydrogenase activity in the presence of particulate matter is not known, and it is postulated that an inhibitor of the 5β reducing enzyme system is associated with the particulate matter.

Reduction of the C-3 ketone has resulted in both the 3α and 3β-hydroxy epimers, the former predominating. This was evidenced after the isolation and identification of II, III, IV, V, VI, X and XIII. The 3α and 3β-hydrogenase systems did not appear to be associated with any specific cell structures, since both types of activity were found in all the homogenate fractions studied. This is in marked contrast to the enzyme systems involved in the reduction of the ring A double bond, the activities of which could be sharply separated. The reduction to both the 3α and 3β forms confirms the reports of other investigators who demonstrated similar con-
version of C₂₁ steroids with rat liver tissue.

Reduction of the C-20 ketone to both the 20α and 20β-hydroxy epimers has been shown by the isolation and identification of VI, VII and VIII. The evidence suggested that the 20 reducing activity was most prominent in the 78,000 x g residue. This, however, did not preclude the absence of the 20-hydrogenase systems in the other homogenate fractions. Since the other homogenate fractions (6000 x g residue, and the particulate-free supernatant) were found to be more active with regards to reduction of the α,β-unsaturated system of ring A, C-20 reduced products may have been present, which had undergone further reduction making their detection on paper chromatograms difficult. Other investigators have reported reduction to either 20α or 20β epimers. This report, therefore, represents the first instance in which the conversion to both epimers has been observed in any one in vitro system.

The isolation and identification of the 6β-hydroxy derivative of 11-deoxycortisol, IX, has unequivocally established the ability of rat liver tissue to hydroxylate the steroid nucleus. The conversion was obtained with the 78,000 x g residue. Whether or not the other homogenate fractions possessed the hydroxylating enzyme systems has not been determined. 6β-Hydroxylation in liver tissue has been indicated by other workers. However, the products were not isolated and crystallized as was the case in this investigation.
Oxidative removal of the α-ketol side chain has been demonstrated for the first time with tissue homogenate fractions. This was shown by the isolation and identification of the four 17-ketosteroids X, XI, XII and XIII. This observation indicates that the intact cell is not essential for the oxidative removal of the side chain. The activity appeared to be present in all the homogenate fractions studied. Employing isolated perfused rat livers a similar conversion has been previously reported by only one group of workers. These investigators were able to isolate 17-ketosteroids when cortisone was perfused through livers.

The results of this investigation have clearly shown the multiplicity of reactions that liver enzyme systems can effect on the steroid molecule. These reactions include reduction of the nuclear double bond, reduction of the C-3 and C-20 carbonyl functions, 6β-hydroxylation and oxidative removal of the α-ketol side chain. Furthermore, it has been possible to demonstrate the following for the first time:

1. The reduction of ring A to both 5α (allopregnane and androstan) and 5β (pregnane and etiocholane) derivatives by rat liver preparations.

2. The reduction of the C-20 ketone function to both the 20α and 20β-hydroxy epimers.

3. The oxidative removal of the C-17 side chain by liver homogenate fractions.
4. Hydroxylation of the steroid nucleus by rat liver preparations.

Based on the results of this present investigation, a tentative step-wise scheme has been proposed for the in vitro metabolism of a typical adrenocortical steroid, 11-deoxycortisol. A major reaction involves reduction of the ring A double bond followed by reduction of the C-3 ketone function. Further reduction can occur at C-20 to form the polyhydroxy non-ketonic compound. However, reduction at C-20 can occur without concomittant reduction of the ring A unsaturation to yield derivatives with glycol type side chains. It is presumed but not proved, that oxidative degradation of the C-17 side chain can take place at any point in the above sequence and any resulting ring A unsaturated C19 steroids thus formed undergo further reduction. Finally hydroxylation at carbon 6 can occur in the liver and has thus far only been shown to take place with the α,β-unsaturation of ring A intact, although hydroxylated ring A-reduced compounds have been indicated.
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APPENDIX
APPENDIX

The writer is indebted to the various commercial concerns and individuals for making available generous samples of reference steroid substances. The sources of the respective reference compounds are as follows:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-deoxycortisol</td>
<td>Merck and Co.</td>
</tr>
<tr>
<td>$\Delta^4$-androstene-3,17-dione</td>
<td>&quot; &quot; &quot;</td>
</tr>
<tr>
<td>17α,21-dihydroxyallopregnane-3,20-dione</td>
<td>Syntex (Mexico)</td>
</tr>
<tr>
<td>3β,17α,21-trihydroxyallopregnane-20-one</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>3β,17α,21-trihydroxypregnan-20-one</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>3β,17α,20β,21-allopregnane-tetrol</td>
<td>T. Reichstein (Switzerland)</td>
</tr>
<tr>
<td>17α,20β,21-trihydroxy-$\Delta^4$-pregnen-3-one</td>
<td>Ciba Pharmaceuticals</td>
</tr>
<tr>
<td>Androsterone</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Androstane-3,17-dione</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Etiocholanolone</td>
<td>Fukushima, D. K. (Sloan-Kettering Institute)</td>
</tr>
<tr>
<td>6β,17α,21-trihydroxy-$\Delta^4$-pregnen-3,20-dione</td>
<td>The Upjohn Co.</td>
</tr>
<tr>
<td>3α,17α,21-trihydroxy-pregnan-20-one</td>
<td>Frank Ungar (Worcester Foundation)</td>
</tr>
</tbody>
</table>

In most instances, the compounds were obtained by partial synthesis using well established organic chemical
reactions leading to unambiguous products. 6β,17α,21-Trihydroxy-Δ^4\textsuperscript{11}-pregnene-3,20-dione was prepared by the microbiological oxidation of 11-deoxycortisol and the structure verified by chemical means. 3α,17α,21-Trihydroxypregnane-20-one was isolated from an incubation of 17α,21-dihydroxy pregnane-3,20-dione. The steric configuration of the product at C\textsubscript{5} was predetermined by the nature of the starting material. The configuration of the hydroxyl at position 3 was established by degradation to the known product etiocholanolone.

The authenticity of the compounds were checked by the writer by melting point determinations, homogeneity and mobility rates in the various paper partition chromatographic systems.
BIOGRAPHY

The author was born January 26, 1918 in West Boylston, Massachusetts, the son of Mariano and Teresa Forchielli. After attending grade schools in West Boylston and Worcester and high school in Worcester, he matriculated to the Massachusetts College of Pharmacy (1936-1940) where he received a B.S. degree in Pharmacy. He attended Clark University from 1949 to 1951 and received an A.B. degree. Following this he entered the Boston University Graduate School in 1951 where he completed formal requirements for the A.M. degree which he received in 1953. The research for the degree was carried on at the Worcester Foundation for Experimental Biology. He then continued on at the Graduate School to fulfill formal requirements for the Doctor of Philosophy degree while performing the research presented in this dissertation at the Worcester Foundation for Experimental Biology.
THE IN VITRO METABOLISM OF A TYPICAL ADRENOCORTICAL STEROID (11-DEOXYCORTISOL)

Abstract of a Dissertation

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BOSTON UNIVERSITY GRADUATE SCHOOL

by

Enrico Henry Forchielli

B.S. in Pharmacy, Massachusetts College of Pharmacy, 1940
A.B., Clark University, 1951
A.M., Boston University, 1953

Department: Medical Sciences
Field of Specialization: Biochemistry
Major Instructor: Professor Ralph I. Dorfman

1956
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V 3β,17α,21-trihydroxypregnan-20-one
VI 3β,17α,20β,21-allopregnanetetrol
VII 17α,20β,21-trihydroxy-Δ4-pregnen-3-one
VIII 17α,20α,21-trihydroxy-Δ4-pregnen-3-one
IX 6β,17α,21-trihydroxy-Δ4-pregnen-3,20-dione
X androsterone
XI androstane-3,17-dione
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Reduction of ring A to the 5α (allopregnane or androstane) derivatives has been demonstrated by the isolation and identification of I, II, III, X and XI whereas reduction to the 5β (pregnane or etiocholane) derivatives has been demonstrated by the isolation and identification of III, IV, and XIII. Other investigators employing either rat liver
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Reduction of the C-3 ketone has resulted in both the 3α and 3β-hydroxy epimers, the former predominating. This was evidenced after the isolation and identification of II, III, IV, V, VI, X and XIII. The 3α and 3β-hydrogenase systems did not appear to be associated with any specific cell structures, since both types of activity were found in all the homogenate fractions studied. This is in marked contrast to the enzyme systems involved in the reduction of the ring A double bond, the activities of which could be sharply separated. The reduction to both the 3α and 3β forms confirms the reports of other investigators who demonstrated
similar conversion of C₂₁ steroids with rat liver tissue.

Reduction of the C-20 ketone to both the 20α and 20β-hydroxy epimers has been shown by the isolation and identification of VI, VII and VIII. The evidence suggested that the 20 reducing activity was most prominent in the 78,000 x g residue. This, however, did not preclude the absence of the 20-hydrogenase systems in the other homogenate fractions. Since the other homogenate fractions (6000 x g residue, and the particulate-free supernatant) were found to be more active with regards to reduction of the α,β-unsaturated system of ring A, C-20 reduced products may have been present, which had undergone further reduction making their detection on paper chromatograms difficult. Other investigators have reported reduction to either 20α or 20β epimers. This report, therefore, represents the first instance in which the conversion to both epimers has been observed in any one in vitro system.

The isolation and identification of the 6β-hydroxy derivative of 11-deoxycortisol, IX, has unequivocally established the ability of rat liver tissue to hydroxylate the steroid nucleus. The conversion was obtained with the 78,000 x g residue. Whether or not the other homogenate fractions possessed the hydroxylating enzyme systems has not been determined. 6β-Hydroxylation in liver tissue has been indicated by other workers. However, the products were not isolated and crystallized as was the case in this investigation.
Oxidative removal of the \( \alpha \)-ketol side chain has been demonstrated for the first time with tissue homogenate fractions. This was shown by the isolation and identification of the four 17-ketosteroids X, XI, XII and XIII. This observation indicates that the intact cell is not essential for the oxidative removal of the side chain. The activity appeared to be present in all the homogenate fractions studied. Employing isolated perfused rat livers a similar conversion has been previously reported by only one group of workers. These investigators were able to isolate 17-ketosteroids when cortisone was perfused through livers.

The results of this investigation have clearly shown the multiplicity of reactions that liver enzyme systems can effect on the steroid molecule. These reactions include reduction of the nuclear double bond, reduction of the C-3 and C-20 carbonyl functions, \( \beta \)-hydroxylation and oxidative removal of the \( \alpha \)-ketol side chain. Furthermore, it has been possible to demonstrate the following for the first time:

1. The reduction of ring A to both 5\( \alpha \) (allopregnane and androstane) and 5\( \beta \) (pregnane and etiocholane) derivatives by rat liver preparations.

2. The reduction of the C-20 ketone function to both the 20\( \alpha \) and 20\( \beta \)-hydroxy epimers.

3. The oxidative removal of the C-17 side chain by liver homogenate fractions.
4. Hydroxylation of the steroid nucleus by rat liver preparations.

Based on the results of this present investigation, a tentative step-wise scheme has been proposed for the *in vitro* metabolism of a typical adrenocortical steroid, 11-deoxycortisol. A major reaction involves reduction of the ring A double bond followed by reduction of the C-3 ketone function. Further reduction can occur at C-20 to form the polyhydroxy non-ketonic compound. However, reduction at C-20 can occur without concomittant reduction of the ring A unsaturation to yield derivatives with glycol type side chains. It is presumed but not proved, that oxidative degradation of the C-17 side chain can take place at any point in the above sequence and any resulting ring A unsaturated C₁₉ steroids thus formed undergo further reduction. Finally hydroxylation at carbon 6 can occur in the liver and has thus far only been shown to take place with the α,β-unsaturation of ring A intact, although hydroxylated ring A-reduced compounds have been indicated.