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Steroid Biochemistry

Volume II

Edited by
Ronald Hobkirk

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Editor

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PREFACE

It was initially anticipated that about eight or nine chapters on various topics in steroid biochemistry would constitute a single volume of this publication. However, it became apparent that the literature in certain of the areas covered was so considerable as to necessitate two volumes, each containing four chapters. Since it was clearly advantageous that these volumes should be of approximately equal length, the original order of chapters had to be altered somewhat. This change, however, has not resulted in any major disadvantage.

An attempt has been made to attract contributions which illustrate the importance of certain enzymatic processes involved in steroid biosynthesis and metabolism and, in some cases, leading to steroidal action in target sites. Investigators actively engaged in research in such areas were invited to present their material in a manner which they considered fitting. It is hoped that as a result of this, the publication will possess sufficient depth to warrant approval. The blend of review material and experimental data originating in the authors' laboratories will, it is felt, make for useful reading.

As is the case with virtually every publication which involves multiple contributors, the various chapters were completed at rather different times, resulting in some being more current than others. For this reason dates of receipt are included in the following brief description of the contents.

In Volume I the chapter by A. F. Clark (July 1978) deals with the reductase enzymes acting at carbon-5 of the steroid molecule, with particular emphasis upon the potential hormonal activities of the products. An in-depth view of the chemistry and biochemistry of steroid carboxylic acids is provided by H. L. Bradlow and C. Monder (November 1977). It should be noted that the original work of these authors provided most of our current knowledge on this topic. D. G. Williamson's review (June 1977) of the biochemistry of 17-hydroxysteroid dehydrogenases focuses upon the role of these enzymes in the biological activation and inactivation of steroids. A. H. Payne and S. S. Singer (July 1977) discuss the role of steroid sulfotransferase and sulfatase systems from a biochemical viewpoint. In addition, they draw attention to the possible biological role of glucocorticoid sulfates. Volume II of the publication contains a chapter by B. R. Bhavnani and C. A. Woolever (August 1977) on biosynthetic pathways and some aspects of metabolism and biological activity of ring B unsaturated estrogens. This is a topic which has been primarily elucidated by the experimental work of Bhavnani. J-G. Lehoux (July 1977) reviews the complex area of the control of mineralocorticoid biosynthesis and the involvement of peptide hormones, electrolytes, prostaglandins, serotonin, etc. P. I. Musey, K. Wright, J. R. K. Preedy and D. C. Collins (July 1978) deal with steroid conjugate formation in relation to steroid distribution in body tissues and fluids. Factors related to the biological hydroxylation of 18- and 19-carbon steroids are discussed in a chapter by R. Hobkirk (May 1977).

It is my distinct pleasure to thank each contributor for the effort put into this publication. I should also like to acknowledge the help accorded me by CRC Press, particularly by Coordinating Editor Benita Segraves.

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THE EDITOR

Dr. R. Hobkirk was born in the town of Peebles in Scotland and attended the local High School. After graduating B.Sc. with Honors in Biochemistry from the University of Edinburgh in 1952 he completed a Ph.D. degree at the same institution in 1955. This latter involved studies on the biochemistry of plant cell wall polysaccharides. The following two years were spent as a research fellow (British Empire Cancer Campaign) in the Departments of Biochemistry and Surgery, University of Glasgow. During that period studies were performed on the relationship of steroid hormones to human breast cancer. Part of the 1957—'58 year was spent as a research fellow in the Department of Biochemistry, McGill University, Montreal and then, until 1960, in the Department of Metabolism, The Montreal General Hospital, Montreal. In 1960 Dr. Hobkirk was appointed Assistant Professor in Experimental Medicine, McGill University, followed in 1962 by promotion to Associate Professor and in 1967 to Professor (Biochemistry) in the Department of Medicine. In 1966 he was appointed a Research Associate of the Medical Research Council of Canada (i.e., a full time investigatorship), an award which he continues to hold. In 1971 he moved to the University of Western Ontario, London, Canada, and is currently Professor in the Department of Biochemistry. He is occupied with research and teaching in hormone biochemistry. In 1973 he received the degree of D.Sc. from the University of Edinburgh for a thesis entitled, "Metabolism of the Estrogens and their Conjugates".

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Chapter 1

ALTERNATE PATHWAYS OF STEROID BIOSYNTHESIS AND THE ORIGIN, METABOLISM, AND BIOLOGICAL EFFECTS OF RING B UNSATURATED ESTROGENS

B. R. Bhavnani and C. A. Woolever

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I. INTRODUCTION

For the past 25 years, it has been generally accepted that cholesterol is the obligatory precursor in the biosynthesis of all steroid hormones. Details of the various steps for the synthesis of cholesterol from acetate and for its conversion to steroids were established mainly by in vitro incubation procedures using tissues such as the ovary, testis, and adrenal cortex. Although it became firmly established that acetate was the initial two carbon source of cholesterol, some experimentation suggested that cholesterol may not be the only precursor from which all steroid hormones are formed.

This chapter will discuss the evidence for the steroidogenic pathway which bypasses cholesterol. It will be referred to as the alternate pathway of steroidogenesis, while that which utilizes cholesterol as an obligatory intermediate will be termed the classical pathway of steroidogenesis. Although it is not the purpose of this chapter to discuss the classical pathway of biosynthesis and metabolism of steroid hormones, its salient features will be reviewed briefly to facilitate comparison with the alternate pathway, which will be discussed in detail. The metabolism and biological action of the ring B unsaturated estrogens will also be presented.

II. CLASSICAL PATHWAY OF STEROIDOGENESIS

The various steps in the classical pathway of steroidogenesis from acetate are acetate → mevalonic acid → isopentenyl pyrophosphate → 3,3-dimethyl allyl pyrophosphate → geranyl pyrophosphate → farnesyl pyrophosphate → squalene → lanosterol → cholesterol → pregnenolone → steroid hormones. The details are discussed below.

A. Source of Carbon Atoms of Cholesterol

Studies by Bloch, Lynen, Cornforth, Popják, and their colleagues on cholesterol biosynthesis from either methyl or carboxyl ^{14}C-labeled acetate established that all 27 carbon atoms of cholesterol are derived from acetate. These investigations have been reviewed by Bloch.[1] The distribution of the 15 methyl and 12 carboxyl carbons in cholesterol is shown in Figure 1. This arrangement of the carbon atoms of acetate in cholesterol follows the "isoprene rule," which was defined by Ruzicka as a working hypothesis as early as 1921.[2]

B. Biosynthesis of Mevalonic Acid

Three molecules of acetic acid in the form of acetyl-CoA (coenzyme A) thioester

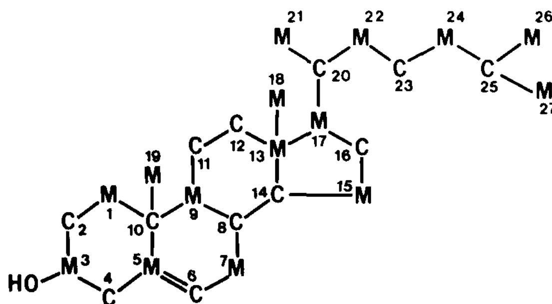


FIGURE 1. Distribution of carboxyl(C) and methyl(M) carbons of acetic acid in cholesterol.

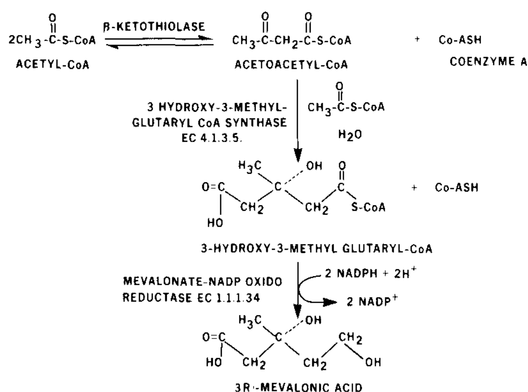


FIGURE 2. Biosynthesis of mevalonic acid from acetyl coenzyme A.

condense to form mevalonic acid (Figure 2). All of the intermediates involved are bound to coenzyme A, and the required enzymes are present in both the cytoplasmic particles and the soluble fraction of mammalian and avian liver[3-6] and in yeast.[7,8] The first step, catalyzed by a soluble enzyme β-keto-thiolase, results in the formation of acetoacetyl-CoA. In the presence of 3-hydroxy-3-methyl-glutaryl-CoA synthase (which is located in the mitochondria and the soluble fraction of liver[9,10] but not in the microsomal fraction as had been reported previously[11,12]), the acetoacetyl-CoA condenses with a third molecule of acetyl-CoA to give 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), the immediate precursor of mevalonic acid. The reduction of this precursor to mevalonic acid is catalyzed by mevalonate: $NADP^+$ oxidoreductase (EC 1.1.1.34), which is present in both yeast mitochondria[13] and in the mammalian liver microsomes.[14,15] This microsomal system is thought to be the rate-limiting step in the biosynthesis of cholesterol in mammalian liver.[12,16,17] Although this scheme is generally believed to be the major pathway of mevalonate biosynthesis, an alternate minor pathway involving malonyl-CoA has been described by Brodie et al.[18,19] The mechanisms involved in these transformations have been recently reviewed by Beytía and Porter.[20]

C. Formation of Isopentenyl Pyrophosphate From Mevalonic Acid

The biosynthesis of isopentenyl pyrophosphate (IPP) from 3R-mevalonic acid is catalyzed by soluble enzymes and involves phosphorylated intermediates. Mevalonic acid is sequentially phosphorylated first (Figure 3) with ATP in the presence of mevalonic

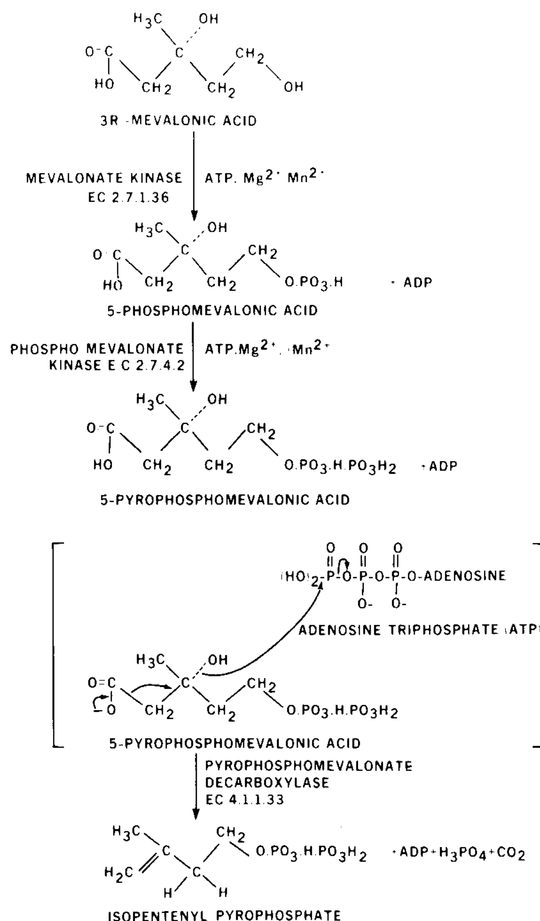


FIGURE 3. Conversion of mevalonic acid to isopentenyl pyrophosphate.

kinase (ATP: mevalonate-5-phosphotransferase, EC 2.7.1.36) and Mg^{2+} or Mn^{2+} ions to 5 phosphomevalonic acid[1,20-26] which, in the presence of phosphomevalonate kinase (ATP: 5-phosphomevalonate phosphotransferase, EC 2.7.4.2), ATP, and Mg^{2+} or Mn^{2+}, is further phosphorylated to 5-pyrophosphomevalonic acid.[1,20,21,26-29] The latter is then decarboxylated and dehydrated to *isopentenyl pyrophosphate* by elimination of the carboxyl and 3-hydroxy (tertiary) groups. The reaction is catalyzed by pyrophosphomevalonate decarboxylase (ATP: 5-pyrophosphomevalonate carboxy-lyase, EC 4.1.1.33) and ATP. It has been suggested that the tertiary hydroxyl group is phosphorylated to form an enzyme-bound intermediate that undergoes concerted *trans* elimination[33] of phosphate and decarboxylation to yield isopentenyl pyrophosphate.[21,26,27,31-33] The ATP is thought to act as a nucleophile attacking the 3-hydroxyl group with the displacement of ADP and formation of inorganic phosphate. The isopentenyl pyrophosphate is the "biological isoprene unit"[2] from which all polyisoprenoid compounds are derived.

D. Formation of Squalene From Isopentenyl Pyrophosphate

Some of the isopentenyl pyrophosphate is rapidly isomerized to dimethylallyl pyrophosphate (DPP) by addition of a proton to the terminal (double bond) methylene carbon, followed by *trans* elimination of a proton from C-2 in a concerted reaction[1,20,21,34] (Figure 4). The enzyme that catalyzes the isomerization of the double

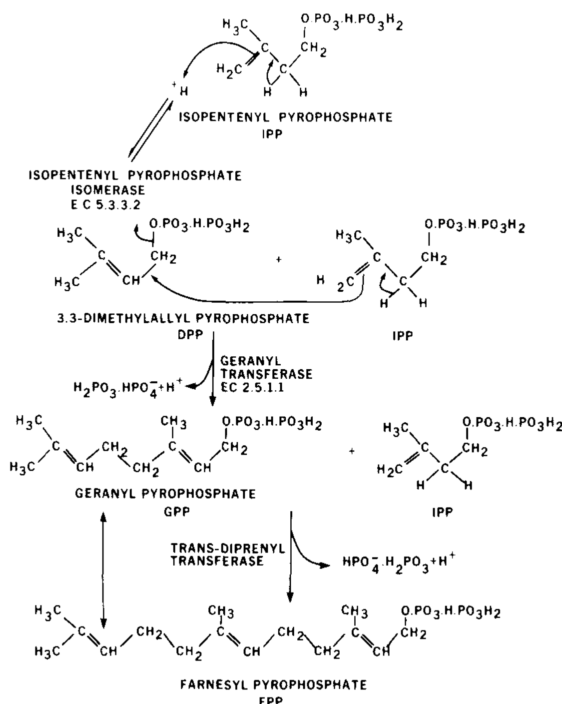


FIGURE 4. Formation of farnesyl pyrophosphate from isopentenyl pyrophosphate.

bond from position 3 of isopentenyl pyrophosphate to position 2 of dimethylallyl pyrophosphate is IPP isomerase (EC 5.3.3.2) and was first isolated from yeast[35] and subsequently from pig liver.[36-38]

The two 5-carbon alcohol pyrophosphates (IPP and DPP) are the biological equivalents of isoprene. Since IPP contains a terminal methylene group and DPP is an allylic pyrophosphate, the two can readily condense with elimination of a pyrophosphate group. This enzymic head to tail or asymmetric condensation occurs by means of a phosphate elimination from DPP with the generation of a carbonium ion, which attacks the terminal methylene group of IPP. Elimination of a proton from carbon-2 of IPP gives rise to geranyl pyrophosphate (GPP) in which the allylic pyrophosphate structure is retained. The enzyme catalyzing this reaction is geranyl transferase (*trans-diprenyl transferase,* EC 2.5.1.1) first described by Lynen et al.,[39] in yeast autolyzates.

The GPP can similarly couple with another molecule of IPP in the presence of farnesyl transferase (C-15-specific prenyl transferase)[37,39,40] to form farnesyl pyrophosphate (FPP) with the allylic pyrophosphate structure still intact (Figure 4).

Repetition of the above head to tail condensations between an allylic pyrophosphate and IPP leads to polyprenyl pyrophosphates up to C-110.[20] The diterpene moiety of carotenoids,[20,41] the side chain of various quinones,[42] and rubber[43] are formed by these condensations. In all instances except rubber, the newly formed bond is *trans* in configuration.

However, when two molecules of FPP in the presence of microsomal squalene synthetase condence tail to tail (Figure 5) in the presence of NADPH, squalene is formed.[20,21,26,30,44,45] It has been shown recently that squalene is not formed directly by the above condensation reaction. Rather, the new intermediate presqualene pyrophosphate (PSPP) is formed, which is subsequently transformed to squalene.[46-50] Formation of PSPP takes place in the absence of NADPH. Thus, condensation of two molecules of FPP in the presence of squalene synthetase and Mg^{2+} results in the loss of

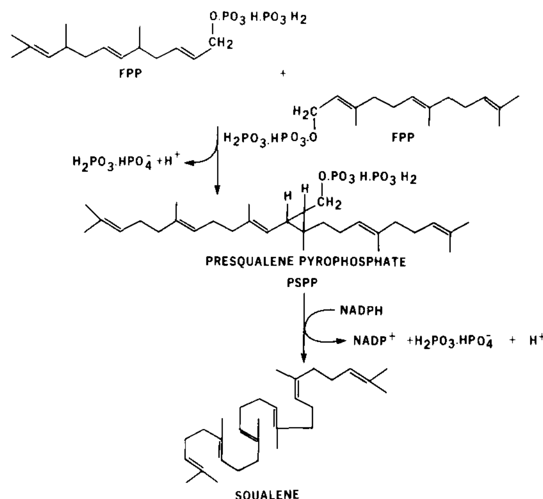


FIGURE 5. Conversion of two molecules of farnesyl pyrophosphate to squalene.

one of the allylic pyrophosphate groups from one FPP (Figure 5). A proton from the carbon-1 of the same FPP is also lost, resulting in a cyclopropane derivative PSPP. The subsequent conversion of PSPP to squalene occurs in the presence of NADPH by a reaction whereby the cyclopropane ring is opened and a pyrophosphate moiety is lost. This is followed by the addition of a hydride ion from NADPH to that central carbon of the squalene molecule which was derived from carbon-1 of the FPP that had lost a proton in the first step.[51] Cornforth[52] has argued against PSPP being a true intermediate in the formation of squalene from two molecules of FPP. He has indicated that (1) PSPP may be an artifact resulting from the absence of NADPH and (2) it is highly unlikely that the same enzyme squalene synthetase can catalyze two different reactions, namely, condensation and reduction. However, Qureshi et al.[49] reported that squalene synthetase exists in two forms: "polymeric" and "protomeric." The polymeric form synthesizes both squalene and PSPP while the protomeric form synthesizes only PSPP. Muscio et al.[53] demonstrated that PSPP is also formed in the presence of NADPH. Thus, it seems that PSPP is indeed a true intermediate in the formation of squalene. The detailed stereochemical mechanisms involved in some of these conversions have been described.[1,20,21,48,50,52,54,55]

E. Cyclization of Squalene to Lanosterol

The cyclization of squalene to lanosterol[56-61] marks the beginning of the aerobic phase of sterol biosynthesis and requires molecular oxygen, NADPH, and liver microsomes. The first reaction in this two-step process (Figure 6) is the production of 2,3-oxidosqualene. This epoxidation of squalene is catalyzed by microsomal squalene epoxidase (mono-oxygenase, EC 1.14.99.7) and NADPH, which brings about incorporation of one atom of "activated molecular oxygen" of unknown identity into 2,3-oxidosqualene. The oxide then undergoes an anaerobic cyclization to lanosterol, catalyzed by squalene oxide cyclase (Figure 6). The cyclization of 2,3-oxidosqualene is postulated to be initiated by attack of a proton on the oxide ring and is followed by a concerted electron shift leading to ring closures and formation of a carbonium ion at carbon -20 of a protosterol. This is followed by a series of 1,2,-*trans* migrations of hydride and methyl groups, resulting in the elimination of a proton from C-9 to give rise to lanosterol (Figure 6).

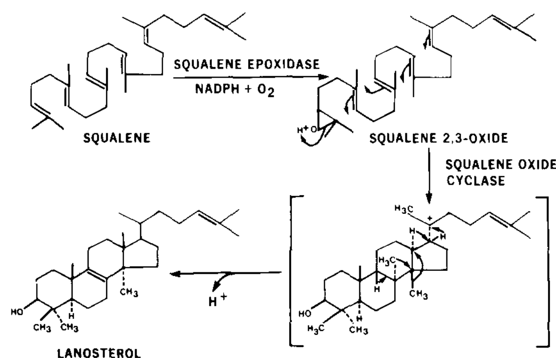


FIGURE 6. Cyclization of squalene to lanosterol.

F. Conversion of Lanosterol to Cholesterol

The exact pathway(s) for the conversion of lanosterol to cholesterol has not been elucidated. Three types of changes occur:

1. The reduction of the Δ^{24}-double bond in the side chain
2. The migration of Δ^{8}-double bond to Δ^{5}
3. The elimination of the three methyl groups at C-4 and C-14

Many potential cholesterol precursors at varying stages of C-4 and C-14 demethylation, and saturation or unsaturation in the side chain have been detected and isolated from natural sources (Figure 7). These have been shown to be converted to cholesterol[62,63] enzymatically. The stereochemistry of these reactions has been elucidated and reviewed by Good.[64]

Although the point in the biosynthetic pathway at which the Δ^{24} bond is saturated has not been resolved, it is known that the reduction of the double bond is achieved by NADPH and a microsomal Δ^{24} reductase.[65] The demethylation at C-4 and C-14 is considered to occur by oxygenation of the methyl groups to an alcohol, followed by oxidation to carboxyl groups (via aldehyde), and finally decarboxylation.[66,67] The C-14 methyl group appears to be the first of the three groups to be removed.[68,69] Recently, it has been shown[70] that the C-14 methyl group is removed as formic acid and not as carbon dioxide. Schroepfer et al.[63] reported the formation of $\Delta^{8(14)}$-monoene sterol as the first product of C-14 demethylation, with subsequent formation of $\Delta^{8(14)}$-diene via the formation of a 15-hydroxylated intermediate.

The formation of the Δ^{5}-double bond of cholesterol has been established to occur via the sequence $\Delta^{8} \rightarrow \Delta^{7} \rightarrow \Delta^{5,7} \rightarrow \Delta^{5}$ (Figure 7).[64] Two possible pathways from lanosterol to cholesterol are summarized in Figure 7; however, other possibilities also exist.

G. Conversion of Cholesterol to Pregnenolone

It is generally accepted now that the C_{21} steroid pregnenolone is the major precursor of steroid hormones in all endocrine tissues capable of *de novo* synthesis (ovary, testis, and adrenal cortex). Pregnenolone is formed enzymatically from unesterified cholesterol by side-chain cleavage between carbon-20 and carbon-22. This conversion involves a series of mixed-function oxidase reactions requiring NADPH and molecular oxygen.

Burstein et al.[71] and Burstein and Gut[72,73] incubated human, guinea pig, and bovine adrenal preparations with hydroxylated cholesterol derivatives and demonstrated the existence of at least two pathways: (1) cholesterol → (22R)-22-hydroxycholesterol → (22R)-20α,22-dihydroxycholesterol → pregnenolone and (2) cholesterol → 20α-hydroxycholesterol → (22R)-20α,22-dihydroxycholesterol → pregnenolone (Figure 8). These

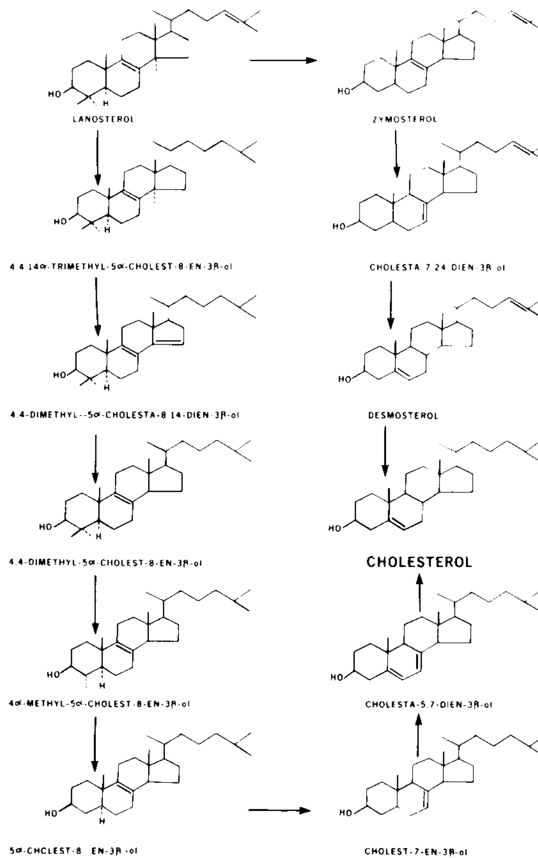


FIGURE 7. Conversion of lanosterol to cholesterol.

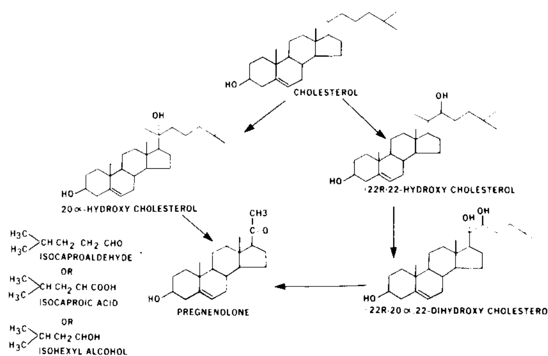


FIGURE 8. Conversion of cholesterol to pregnenolone.

authors also indicated that (22R)-22-hydroxycholesterol and 20α,22R-dihydroxycholesterol were better substrates than 20α-hydroxycholesterol. They further indicated that these two pathways accounted for a relatively small part of the experimentally formed pregnenolone and that most of the pregnenolone arose from several direct (or one-step) enzymatic reactions from cholesterol to (22R)-20α,22-dihydroxycholesterol.

Burstein and Gut[73] have shown that preequilibration of substrate with the enzyme prior to starting the reaction (by adding NADPH) gave polyphasic kinetics with higher reaction rates. These results indicate the existence of a compartmentalized system for

the conversion of cholesterol to pregnenolone in adrenal preparations. Whether these increased reaction rates could account for all of the pregnenolone formed by a series of hydroxylations and side-chain cleavage reactions remains to be established. Some of these results could also be explained on the basis that other intermediates, such as cholesterol 20α-hydroperoxide,[74] may be involved in the formation of pregnenolone. Similarly, Lieberman et al.[75] have postulated the existence of transient free radical (or ionic) intermediates in the conversion of cholesterol to pregnenolone. Recently, Luttrell et al.[76] showed that (20R)-20-t-butyl-5-pregnene-3β,20-diol can be converted to pregnenolone, indicating that enzymatic reactions in this conversion could involve free radical intermediates. However, the presence of such intermediates in the normal conversion of cholesterol to pregnenolone remains to be established.

The nature of the C_6 compounds formed during the side-chain cleavage of cholesterol appears to depend on the species. It seems that the initial product formed is isocaproaldehyde (Figure 8), which is either reduced to isohexylalcohol or oxidized to isocaproic acid.[72,77,78] Burstein and Gut[72] further showed that the stoichiometric relationship between pregnenolone and the C_6 compounds was approximately 1:1. This indicates that the cholesterol side-chain hydroxylations occur mainly at position C-20 and C-22, prior to side-chain cleavage.

Jungmann[79,80] reported that cholesterol can be directly converted to the C_{19} steroid dehydroepiandrosterone without the formation of pregnenolone. He indicated that during the side-chain cleavage of cholesterol a C_8 compound 2-methyl-6-heptanone was formed. However, Burstein et al.[81] and Hochberg et al.[82] were unable to confirm unequivocally that 2-methyl-6-heptanone was formed from cholesterol. Therefore, the possibility of an alternative pathway producing C-19 steroids by direct side-chain cleavage remains to be established.

Pregnenolone is subsequently metabolized to other steroids, resulting in the formation of gestagens (progestins), corticosteroids, androgens, and estrogens.

III. ALTERNATE PATHWAYS OF STEROIDOGENESIS

Research reviewed in the preceding section indicates very clearly that cholesterol is firmly established as a key precursor of steroids formed via the classical pathway. This section will discuss evidence for the existence of alternate pathways of steroidogenesis that do not require cholesterol as the obligatory intermediate.

Hechter[83] had speculated nearly 2 decades ago that there were pathways of corticosteroid biosynthesis which bypass cholesterol and suggested the involvement of compounds with 20, 22, and 24 carbons in the formation of C_{19}, C_{21}, C_{24}, and C_{27} steroids. Heard et al.[84] administered (1-^{14}C) acetate to a pregnant mare and isolated ^{14}C-labeled estrone, equilin, and equilenin from the urine. The molar specific activity of estrone was almost twice that of equilin and equilenin. Based on these findings, Dorfman[85] speculated that ring B unsaturated estrogens, equilin and equilenin, may be derived by the condensation of an ethyl toluene residue with a carbon-10 isoprenoid unit (formed from acetate). No experimental data were presented, nor was this hypothesis ever tested experimentally.

A. Direct Conversion of Desmosterol to Steroid Hormones

Goodman et al.[86] demonstrated that desmosterol, an immediate precursor of cholesterol (Figure 7), was directly transformed to steroid hormones and bile acid in humans treated with triparanol (1-(p-β-diethylaminoethoxyphenyl)-1-(p-tolyl)-2-(p-chlorophenyl) ethanol), a drug which inhibits the reduction of the Δ^{24} bond of desmosterol.

Similarly, Goodman et al.[87] demonstrated that ^{14}C-desmosterol was directly metabolized to corticosteroids by adrenal preparations from triparanol-fed rats and mice.

Hall[88] also suggested that desmosterol instead of cholesterol was a precursor of testosterone in testis of rabbits which had been pretreated with triparanol.

Shimizu and Gut[89] extended these investigations and demonstrated that incubation of cholesta-5,24-dien-3β-ol-26^{14}C (desmosterol) (Figure 9) with calf adrenal preparations gave rise to pregnenolone and ^{14}C-4-methyl-3-pentenoic acid. These results indicate that (1) the side-chain cleavage of demosterol at positions C-20 and C-22 could take place without the prior reduction of the Δ^{24}-double bond and (2) desmosterol can act as a direct precursor for pregnenolone without first being converted to cholesterol.

Desmosterol is present in man and other species in very low concentrations as compared to cholesterol and therefore probably plays a minor role in normal steroid hormone production by the above pathway.

B. Sesterterpene Pathway of Steroidogenesis

Tait[90-92] suggested that steroid hormone biosynthesis could proceed via a C_{25} terpene (sesterterpene) without the involvement of cholesterol (Figure 10). He incubated 7-^{3}H-23,24-dinor-5-cholen-3β-ol with canine adrenal homogenates[92] and isolated small amounts of radiochemically pure cortisol (Figure 11). Tait[90] further showed that 7-^{3}H-

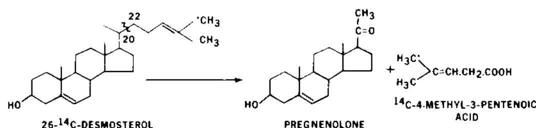


FIGURE 9. Direct conversion of 26-^{14}C*-desmosterol to pregnenolone.

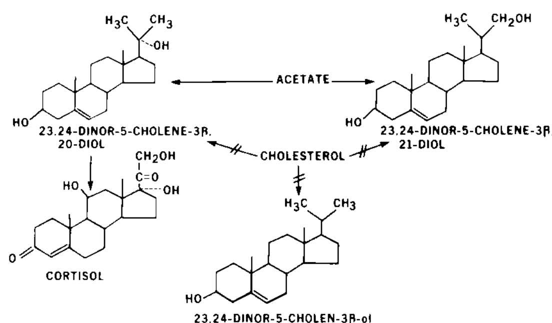


FIGURE 10. Sesterterpene pathway of steroidogenesis.

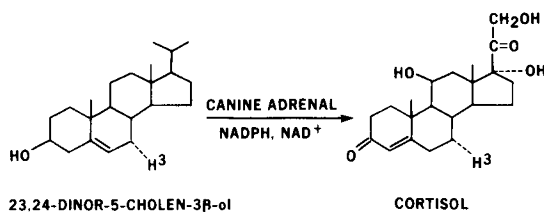


FIGURE 11. Conversion of 7-^{3}H-23,24-dinor-5-cholen-3β-ol to cortisol by canine adrenal homogenates.

23,24-dinor-5-cholen-3β-ol was metabolized to ^{3}H-23,24-dinor-5-cholene-3β,21-diol and ^{3}H-23,24-dinor-5-cholene-3β,20-diol by bovine adrenal homogenates and that the latter steroid was subsequently metabolized to pregenolone, progesterone, 17α-hydroxypregnenolone, corticosterone, and cortisol. Additionally, Tait and Mills[93] reported that ^{3}H-23,24-dinor-5-cholen-3β-ol was metabolized to cortisol, corticosterone, and 11-deoxycorticosterone by human adrenal adenomata. These tissue preparations were also capable of transforming ^{3}H-acetate to ^{3}H-23,24-dinor-5-cholene-3β,21-diol. Similarly, Tait[91] had shown that ^{3}H-acetate was transformed by bovine adrenal homogenates to 23,24-dinor-5-cholene-3β,20-diol and 23,24-dinor-5-cholene-3β,21-diol. However, labeled 23,24-dinor-5-cholene,3β-ol was not isolated. Interestingly, these three steroids were not formed from ^{3}H-cholesterol by adrenal preparations (Figure 10).

These results indicate that adrenal preparations are capable of synthesizing *de novo* compounds such as 23,24-dinor-5-cholene-3β,20-diol and 23,24-dinor-5-cholene-3β,21-diol from acetate, but not from cholesterol, and that these 23,24-dinor cholene derivatives can be transformed to steroid hormones. Therefore, at least in the adrenal gland, a pathway of steroidogenesis in which cholesterol is not an obligatory intermediate may exist.

At present, these results are difficult to reconcile with the following observations

1. Burstein et al.[94] had previously reported that bovine adrenal mitochondria converted 23,24-dinor-5-cholene-3β,20-diol to 23,24-dinor-4-cholen-20-ol-3-one, but not to either pregnenolone or progesterone, indicating that no side-chain cleavage took place.
2. 23,24-Dinor-5-cholen-3β-ol was not formed from acetate even though it was transformed to steroid hormones.[91]
3. None of the three 23,24-dinor cholene derivatives have been isolated from any biological fluid, and it is not known whether these in vitro transformations also occur in vivo.

Until some of these questions are answered, the existence of this independent pathway of steroidogenesis remains speculative.

C. Biosynthesis of Ring B Unsaturated Estrogens

The ring B unsaturated estrogens, equilin and equilenin, and their 17-reduced metabolites (Figure 12) are found only in the urine of the pregnant mare. Although they were first described by Girard et al.[95,96] in 1932, their biosynthetic origin remains a mystery. Gaudry and Glen[97] showed that ring B unsaturated estrogens first appear in mare urine about the 4th month of pregnancy and then increase in amount until parturition, at which time they represent about 65% of the total urinary estrogens. Savard[98] studied individual mares from the 4th month of gestation to term. His data were essentially similar to those of Gaudry and Glen:[97] very little equilin was present earlier than the 4th month. As the level of total estrogens increased in the urine, the proportion of equilin also increased, eventually constituting as much as 50% of the total. This increase in production of ring B unsaturated estrogen with advancing gestational age suggests that the feto-placental unit is involved in their biosynthesis, in a manner analogous to the formation of estriol in human pregnancy as described by Diczfalusy.[99,100]

The early work regarding the origin of ring B unsaturated estrogen is summarized in Table 1. Following the administration of 1-^{14}C-acetate (53.6 mCi) into the jugular vein of a pregnant mare, Heard et al.[84,101] isolated ^{14}C-labeled estrone, equilin, and equilenin from the urine. However, in a similar type of experiment, Heard and O'Donnell[102] reported that 4-^{14}C-cholesterol (50 μCi) administered to a pregnant mare

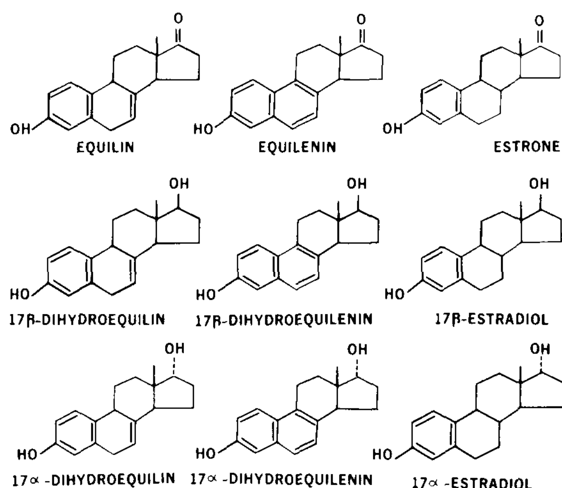


FIGURE 12. Estrogens present in pregnant mares' urine.

TABLE 1

Estrogens Isolated from the Urine of the Pregnant Mare Following Injection of ^{14}C-Labeled Precursors into the Jugular Vein of the Mare

Precursor injected	^{14}C-Labeled estrogen isolated	Nonlabeled estrogen isolated	Ref.
Acetate	Estrone Equilin Equilenin	—	84, 101
Cholesterol	—	Estrone Equilin Equilenin	102
Estrone	Estrone Estradiol	Equilin Equilenin	84
Testosterone	Estrone	Equilin	103
Dehydroepiandrosterone sulfate	Estrone	Equilin Equilenin	105
Δ^7-Dehydroepiandrosterone sulfate	—	Estrone Equilin	105
Estrone sulfate	Estrone 17α-Estradiol 17β-Estradiol	Equilin Equilenin	105

was not a precursor of these estrogens. After 4-^{14}C-testosterone (57 μCi), estrone, but not the ring B unsaturated estrogens (equilin and equilenin) isolated from the urine, was labeled.[103] The conversion of testosterone to estrone in the pregnant mare prompted Heard et al.[103] to reevaluate their negative results reported with cholesterol. They suggested that perhaps the amount of ^{14}C-cholesterol injected into the jugular vein was not enough to allow for dilution with the large pool of endogenous cholesterol which they estimated to be approximately 375 g. Thus, a small conversion of this low specific activity cholesterol will result in estrone essentially devoid of radioactivity and might explain why the estrogens isolated in their study were not radioactive.

When 16-^{14}C-estrone was injected into the jugular vein of a pregnant mare, the equilin and equilenin isolated from the urine were not radiolabeled[84], nor did the

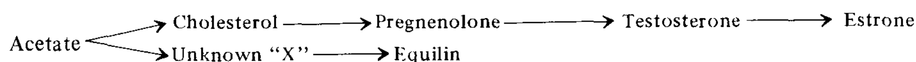
administration of ^{14}C-17β-dihydroequilenin to the pregnant mare result in the formation of labeled estrone or equilin while ^{14}C-labeled equilenin was isolated from this experiment.[104]

These findings indicate that:

1. Ring B unsaturated estrogens in the pregnant mare are not formed from cholesterol.
2. The C_{19} steroids such as testosterone are metabolized to estrone and estradiol but not to the ring B unsaturated estrogens, equilin and equilenin.
3. Estrone is not converted to equilin or equilenin by ring B dehydrogenation; therefore, the ring B unsaturated estrogens are not peripheral metabolites of the classical estrogens.
4. The ring B unsaturated estrogens are not converted to ring B saturated estrogens estrone or estradiol.
5. The ring B unsaturated estrogens equilenin and 17β-dihydroequilenin are not transformed to equilin or 17β-dihydroequilin.
6. Both the classical and the ring B unsaturated estrogens are formed from acetate.

Bhavnani et al.[105] injected a series of ^{14}C-labeled steroid sulfates into the jugular vein of a pregnant mare, anticipating that the sulfates would enter the feto-placental circulation and serve as precursors of the ring B unsaturated estrogens. In these studies (Table 1), 4-^{14}C-dehydroepiandrosterone sulfate was metabolized to estrone but not to equilin or equilenin, 4-^{14}C-Δ^7-dehydroepiandrosterone sulfate was not converted to any known estrogen, and ^{14}C-estrone sulfate was metabolized to ^{14}C-labeled estrone, 17β-estradiol, and 17α-estradiol. These results suggested that the ring B unsaturated estrogens are formed by pathways which are different from the classical pathway of steroidogenesis.

Since both types of estrogens arise from acetate, one can speculate that ring B unsaturated estrogens are formed by a cholesterol-independent pathway involving unknown intermediates as shown below.



Starka et al.[106] perfused human placenta with Δ^7-androstenedione, Δ^7-dehydroepiandrosterone, and 7α-hydroxydehydroepiandrosterone and reported the conversion of these precursors to equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin. The same group[107] also found equilin and equilenin after incubation of 7α-hydroxydehydroepiandrosterone, its 3-sulfate, and Δ^7-dehydroepiandrosterone with human placental tissue cultures. Starka and Breuer[108] reported the formation of equilin from 7α-hydroxydehydroepiandrosterone via Δ^7-dehydroepiandrosterone, and Δ^7-androstenedione by horse placental preparations.

Similarly, Givner et al.[109] demonstrated that a microsomal fraction of human placenta can aromatize Δ^7-steroids such as Δ^7-testosterone, $\Delta^{4,7}$-androstadien-19-ol-3,17-dione, Δ^7-dehydroepiandrosterone to ring B unsaturated estrogens, although it cannot introduce a Δ^7-double bond into estrogens or their Δ^4 or Δ^5-C_{19} precursors. This latter finding is further supported by earlier work of Ainsworth and Ryan,[110] who had shown that horse placenta was incapable of transforming ^{3}H-dehydroepiandrosterone and ^{14}C-androstenedione to ring B unsaturated estrogens. Similarly, Starka et al.[111] had reported that 19-hydroxytestosterone, 19-oxoandrostenedione, and testosterone were not metabolized to ring B unsaturated estrogens by horse placental preparations.

One can speculate from these results that ring B unsaturated estrogens may be formed in vivo from Δ^7-dehydrocholesterol, i.e., part of these Δ^7-sterols are trans-

formed to cholesterol, and a part may follow the Δ^7 pathway to steroids such as Δ^7-dehydroepiandrosterone, which can then be aromatized by the placenta (Figure 13). However, as mentioned above, Δ^7-dehydroepiandrosterone sulfate was not transformed to ring B unsaturated estrogens by the pregnant mare in vivo ruling out this pathway.

Thus, these in vitro studies with the appropriate preformed precursors point only to the availability of an aromatizing enzyme system in human and horse placenta and have little bearing on the in vivo formation of equilin and equilenin by the pregnant mare. It is also important to note that ring B unsaturated estrogens have never been isolated from human pregnancy urine.

There are at least two possible explanations for these negative results of in vivo experiments where the radiolabeled precursor was injected into the jugular vein of a pregnant mare (1) the radiolabeled precursors were excessively diluted in the maternal compartment, and (2) the transplacental passage of precursors into the fetus was hindered because the horse epitheliochorial placenta is a particularly "thick" barrier.[112]

To overcome the problems of placental passage (and knowing that, in the human, estrogens are formed by the fetoplacental unit)[99,100] we developed laparotomy techniques to inject radiolabeled precursors into the fetus. The procedure used in these experiments is as follows:

1. A laparotomy is performed on a mare near term (9 to 10 months), and a mixture of radiolabeled precursor is injected either intravenously (umbilical cord) or intramuscularly into the horse fetus.
2. Following surgery, the mare is allowed to stand.
3. Maternal urine is then collected for 4 to 5 days through a Foley catheter inserted into the bladder.
4. Steroid conjugates present in the urine are either extracted directly with organic solvents or by absorption and elution on a 2-kg Amberlite® XAD-2 resin column.
5. The steroid conjugates are hydrolyzed by solvolysis and β-glucuronidase.
6. The "sulfates" and "glucuronides" are separated into phenolic and neutral fractions by partition between benzene and sodium hydroxide.
7. The fractions are purified by chromatography: column, paper, silica gel thin layer chromatography and Sephadex® LH-20.
8. The metabolites are isolated and identified by melting point, mixed melting point, infrared spectroscopy and mass spectroscopy.
9. Radiochemical purity is established by crystallization to constant specific activity both before and after the formation of a suitable derivative (Tables 2 and 3).

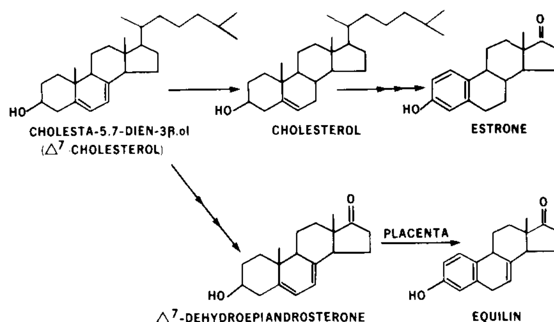


FIGURE 13. Speculative pathway of biosynthesis of equilin from Δ^7-steroids.

TABLE 2

Proof of Radiochemical Purity of Phenolic Metabolites Isolated after Injection of ^{14}C-Mevalonic Acid and ^{3}H-Dehydroepiandrosterone into the Umbilical Circulation of a Horse Fetus

	Specific activity dpm/μmol							
	Equilin		Equilin 3-acetate		Equilin 3-acetate 7α, 8α-glycol		Equilin 7α-8α-glycol	
Crystallization	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
1	110	940	0	200	0	200	0	170
2	50	840	0	200	0	205	—	—
3	0	830	0	200	0	—	—	—
Calculated	470	1,340	0	200[a]	0	220	0	180

	Estrone		Estrone 3-acetate	
	^{3}H	^{14}C	^{3}H	^{14}C
1	29,700	22,600	1,750	1,340
2	29,700	22,600	1,750	1,340
3	29,700	22,600	1,750	1,340
Calculated	19,000	13,800	1,620[a]	1,250[a]

	Equilenin		Equilenin 3-acetate	
	4^{3}H	^{14}C	^{3}H	^{14}C
1	0	60	0	20
2	0	40	0	20
3	0	40	0	—
Calculated	0	80[a]	0	20[a]

	17α-Estradiol		17α-Estradiol diacetate	
	^{3}H	^{14}C	^{3}H	^{14}C
1	380	200	330	200
2	350	190	340	200
3	360	190	340	200
Calculated	450[a]	200[a]	360	190

[a] Isolated material diluted with carrier steroid prior to crystallization or acetylation.

A flow chart for the extraction of steroids from pregnant mares' urine is shown in Figure 14. In the earlier experiments, the urine was extracted directly with methylene dichloride to remove the unconjugated steroids. The aqueous fraction was solvolysed, etc., as shown in Figure 14. The XAD-2 step was not used in some early experiments.

1. Metabolism of 7-^{3}H-Dehydroepiandrosterone and 4-^{14}C-Androstenedione Injected into the Umbilical Vein

A total of 4.3×10^{8} dpm of ^{3}H-dehydroepiandrosterone (specific activity 500 mCi/mmol) and 9.4×10^{7} dpm of ^{14}C-androstenedione (specific activity 34.8 mCi/mmol) was injected into the umbilical vein of a horse fetus.[113] The urine was collected for 4 days and found to contain 3.62×10^{8} dpm of ^{3}H (87%) and 3.7×10^{6} dpm of ^{14}C (41%). It was processed as described in Figure 14, and the distribution of radioactive

TABLE 3

Proof of Radiochemical Purity of Neutral Metabolites Isolated after Injection of ^{14}C-Squalene and ^{3}H-Dehydroepiandrosterone into the Umbilical Circulation of a Horse Fetus

	Specific activity dpm/μmol			
	3β-Hydroxy-5α-pregnan-20-one		3β-acetoxy-5α-pregnan-20-one	
Crystallization	^{3}H	^{14}C	^{3}H	^{14}C
1	0	440	0	550
2	0	450	0	540
3	0	450	0	540
Calculated	0	350	0	520
	5α-Pregnane-3β, 20β-diol		5α-Pregnane-3β,20β-diacetate	
	^{3}H	^{14}C	^{3}H	^{14}C
1	20	290	0	330
2	0	300	0	330
3	0	320	0	320
Calculated	20	290	0	320
	5α-Pregnane-3β,20α-diol		5α-Pregnane-3β,20α-diacetate	
	^{3}H	^{14}C	^{3}H	^{14}C
1	0	340	0	140
2	0	340	0	130
3	—	—	0	140
Calculated	40	300[a]	0	120[a]

[a] Isolated material diluted with carrier prior to crystallization of acetylation.

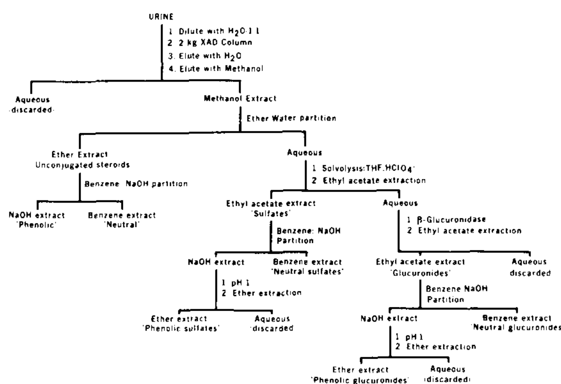


FIGURE 14. Scheme for the extraction of steroids from urine.

material in various fractions is shown in Table 4. Most of the radioactivity recovered was present in the phenolic sulfate fraction (66% of ^{3}H and 58% of ^{14}C). The remaining fractions contained much smaller amounts of radioactive materials. Estrone,

TABLE 4

Distribution of Radioactivity in Various Fractions Following Injection of (7-)^{3}H-Dehydroepiandrosterone and (4)-^{14}C-Androstenedione into the Umbilical Circulation of a Horse Fetus

Fraction	Radioactivity recovered in urine (%)			
	Phenolic		Neutral	
	^{3}H	^{14}C	^{3}H	^{14}C
Unconjugated	0.6	0.4	0.2	0.3
Sulfates	66.0	58.0	4.8	0.3
Glucuronides	6.1	13.0	1.8	4.3

Note: Crude urine ^{3}H = 3.6×10^8 dpm; ^{14}C = 0.37×10^8 dpm

equilin, 17α-estradiol, 17β-estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin were isolated and identified from the phenolic sulfate fraction (Table 5). Only the classical estrogens (i.e., estrone, 17α-estradiol, and 17β-estradiol) contained both ^{3}H and ^{14}C, and their radiochemical purity was established by crystallization to constant specific activity. Thirty percent of ^{3}H-dehydroepiandrosterone and fourteen percent of ^{14}C-androstenedione were converted to estrone, indicating that ^{3}H-dehydroepiandrosterone is a better precursor than androstenedione in the formation of the classical estrogens in this species. It is not clear at present why the two labeled precursors should have such different metabolic fates in the pregnant mare, since in vitro horse placental microsomal preparations convert equal amounts of androstenedione and dehydroepiandrosterone to estrone and estradiol.[110]

In contrast to these findings, the ring B unsaturated estrogens (i.e., equilin, equilenin, 17α-dihydroequilin, and 17α-dihydroequilenin) were devoid of any radioactivity.

These results are in agreement with the demonstration that dehydroepiandrosterone and androstenedione,[110] and testosterone, 19-hydroxytestosterone, and 19-oxoandrostenedione[111] are not converted to equilin or equilenin by horse placental preparations. The data indicate that ring B unsaturated estrogens are formed by pathways independent of the formation of estrone and estradiol.

It is important to note that the dehydroepiandrosterone used was labeled with tritium at the carbon-7 position, and the possibility exists that tritium at this position may have been displaced during the formation of the Δ^7-double bond of equilin, thus giving the negative results. This seems to be highly unlikely as it has been reported[114] that, when 7-^{3}H-dehydroepiandrosterone or 7-^{3}H-cholesterol was synthesized by the reductive dehalogenation of the corresponding 7α-bromo steroids with tritium, the tritium was present at C-3, C-4, C-6, and C-7 positions, respectively. The distribution of this tritium in dehydroepiandrosterone and cholesterol is given in Table 6. It is reasonable to assume that all of the tritium present at position C-7 would not be lost during the formation of the Δ^7-double bond (provided a 7-keytone is not formed). If the pathways for the formation of the estrone and equilin were the same, then even if only 10% of the tritium present in the estrone were left in the equilin, it would have been detected easily.

2. Metabolism of 1-^{14}C-sodium Acetate and 7-^{3}H-Cholesterol Injected into the Fetal Circulation

Since Heard and O'Donnell[102] had reported that cholesterol injected into the jugular vein of the pregnant mare was not transformed to any estrogen, Bhavnani et al.[115]

TABLE 5

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{3}H-Dehydroepiandrosterone and ^{14}C-Androstenedione into the Umbilical Circulation of a Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	^{14}C	Ratio ^{3}H:^{14}C	Weight (mg)
Estrone	440,000	50,000	9.7	88.0
Equilin	0	0	—	28.0
Equilenin	0	0	—	1.0
17α-Estradiol	501,290	68,600	9.1	0.5
17β-Estradiol[a]	—	—	10.7	trace
17α-Dihydroequilin	0	0	—	2.6
17α-Dihydroequilenin	0	0	—	1.6

[a] Due to small amounts isolated, the endogenous specific activity was not determined.

TABLE 6

Distribution of Tritium in 7-^{3}H-steroids

Compounds	Percent of total tritium in position: 3 + 4 + 6	7	7α	7β
Dehydroepiandrosterone-7α-^{3}H (Lot A)	9.4	89	45	44
Dehydroepiandrosterone-7α-^{3}H (Lot B)	16.0	84	82	1
Cholesterol-7α-^{3}H	10.0	86	26	60

reinvestigated the precursor role of cholesterol and acetate. A total of 2 mCi of 1-^{14}C-sodium acetate (specific activity 40 mCi/mmol) and 0.5 mCi of 7-^{3}H-cholesterol (specific activity 500 mCi/mmol) was injected into the fetal circulation and 10% of ^{3}H and 1.4% of ^{14}C was excreted in the urine in 3½ days. The distribution of radioactive material in various fractions is shown in Table 7. The phenolic sulfate fraction which contained 9.6×10^{6} dpm of ^{3}H and 2.0×10^{6} dpm of ^{14}C and from it estrone, equilin, equilenin, 17α-estradiol, 17α-dihydroequilin, and 17α-dihydroequilenin were isolated and identified and their radiochemical purity was established. All the estrogens isolated and identified (Table 8) contained ^{14}C, indicating that they are formed from acetate. Similar results were obtained when acetate was injected into the jugular vein of a pregnant mare.[116] In contrast to these findings, only estrone and 17α-estradiol contained the tritium label (Table 8), while the ring B unsaturated estrogens (i.e., equilin, equilenin, 17α-dihydroequilin, and 17α-dihydroequilenin) were devoid of any tritium.

The data indicates that cholesterol is a precursor of the classical estrogens in the pregnant mare, contrary to the previously reported findings of Heard and O'Donnell.[102] Since the classical estrogens estrone and 17α-estradiol have similar specific activities, it is quite possible that both are derived from acetate by the same pathway.

Ring B unsaturated estrogens have ^{14}C-specific activities which are approximately 30% lower than those of the classical estrogens (Table 8), suggesting that these estro-

TABLE 7

Distribution of Radioactivity in Various Fractions Following Injection of ^{14}C-Sodium Acetate and ^{3}H-Cholesterol into the Umbilical Circulation of a Horse Fetus

Fraction	Radioactivity excreted in urine (%)			
	Phenolic		Neutral	
	^{3}H	^{14}C	^{3}H	^{14}C
Unconjugated total[a]	0.73	0.78	—	—
Sulfates	8.73	3.20	3.27	3.5
Glucuronides	0.53	0.05	0.66	1.1

[a] The unconjugated steroids were not fractionated into phenolic and neutral fractions.

Note: Crude urine ^{3}H = 110 × 10^{6}dpm; ^{14}C = 63 × 10^{6}dpm

TABLE 8

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{14}C-Sodium Acetate and ^{3}H-Cholesterol into the Umbilical Circulation of a Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	Specific activity (dpm/μmol) ^{14}C	Ratio ^{3}H:^{14}C	Weight (mg)
Estrone	1770	1430	1.2	117.0
17α-Estradiol[a]	1470	1440	1.02	2.0
Equilin	0	1040	—	135.0
Equilenin[a]	0	780	—	1.2
17α-Dihydroequilenin[a]	0	1180	—	1.0

[a] Due to small amounts of material isolated, these values are approximate and were calculated from the final specific activity and the carrier added.

gens are derived from acetate (perhaps by a different route) and that the lower specific activities may be due to fewer labeled carbons in molecule. Since there are a number of unknown factors, such as total body pools, compartmentalization, and turnover rates, which may influence the specific activity of a urinary metabolite, the exact nature of the difference in the formation of these estrogens must remain in the realm of speculation. Similar differences in specific activities have been previously reported.[116] Degradation studies are necessary before the exact difference (if any) in the pattern of labeling of carbons in these ring B unsaturated estrogens can be established.

Like the 7-^{3}H-dehydroepiandrosterone used in the previous experiment, the cholesterol used in the above experiment was also labeled at the carbon-7 position. To exclude the unlikely possibility that all of the tritium might have been lost during the introduction of the double bonds if the ring B unsaturated estrogens were formed from cholesterol, and to remove any doubts regarding the conclusion that cholesterol is not a precursor for ring B unsaturated estrogens, its precursor role was reinvestigated by the administration of 4-^{14}C-cholesterol (500 μCi) into a horse fetus.[117] Equilin isolated from the maternal urine was devoid of any radioactivity while estrone contained ^{14}C-labeled material, confirming the results obtained previously with ^{3}H-cholesterol.

These results indicate that ring B unsaturated estrogens are formed by pathways different from the formation of the classicial estrogens, and a bifurcation in the pathway must occur at a point prior to the formation of cholesterol. This demonstration is the first example of in vivo steroidogenesis where cholesterol is not an obligatory intermediate.

3. Metabolism of ^{14}C-Squalene and ^{14}C-Mevalonic Acid Injected into the Umbilical Circulation

To investigate where the bifurcation from the classical pathway occurs, ^{14}C-mevalonic acid and ^{14}C-squalene were administered to pregnant mares via the umbilical vessels.

In the first experiment,[118] (1,5,9,16,20, 24-^{14}C)-squalene (4.5×10^8 dpm, specific activity 28 mCi/mmol) and 7-^{3}H-dehydroepiandrosterone (32.0×10^6 dpm, specific activity 10.4 Ci/mmol) were injected into the fetal circulation; 89% of ^{3}H and 1.24% of ^{14}C was recovered in the maternal urine in 4 days. The phenolic fraction contained 12.8×10^6 dpm of ^{3}H and 2.9×10^6 dpm of ^{14}C, and the neutral fraction contained 2.8 $\times 10^6$ dpm of ^{3}H and 3.1×10^6 dpm of ^{14}C.

Estrone, 17α-estradiol, equilin, equilenin, 17α-dihydroequilin, and 17α-dihydroequilenin were isolated from the phenolic fraction. The classical estrogens, i.e., estrone and 17α-estradiol, were radioactive (Table 9) and contained both ^{3}H and ^{14}C. The ring B unsaturated estrogens were devoid of any radioactivity. It follows that ^{14}C-squalene like cholesterol is not a precursor of the ring B unsaturated estrogens.

The ^{14}C-squalene was prepared enzymatically by incubating rat liver 10,000 × *g* supernatant with dl-2-^{14}C-mevalonic acid and has been shown[119,120] to contain six labeled carbons at positions, 1,5,9,16,20, and 24 of the squalene molecule (Figure 15). The predicted labeling of steroids formed by cyclization of this ^{14}C-squalene biosynthesized from 2-^{14}C-mevalonic acid is depicted in Figure 15. Thus, all of the neutral and phenolic steroids formed by the classical pathway of steroid biosynthesis (i.e., acetate → squalene → cholesterol → pregnenolone → androgens → estrogens) would theoretically contain three labeled carbons at positions 1,7, and 15 (Figure 15). This is based on the fact that the distribution of labeled carbons in both neutral and phenolic steroids is

TABLE 9

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{14}C-Squalene and ^{3}H-Dehydroepiandrosterone into a Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	^{14}C	Weight (mg)
Equilin	2	2	80
Estrone	17,320	205	90
Equilenin	0	0	2
17α-Estradiol[a]	11,970	120	5
3β-Hydroxy-5α-pregnan-20-one	0	450	900
5α-Pregnane-3β, 20β-diol	0	320	210
5α-Pregnane-3β, 20α-diol	0	340	20

[a] Due to small amounts isolated, these values are approximate and were calculated from the final specific activity and the carrier added.

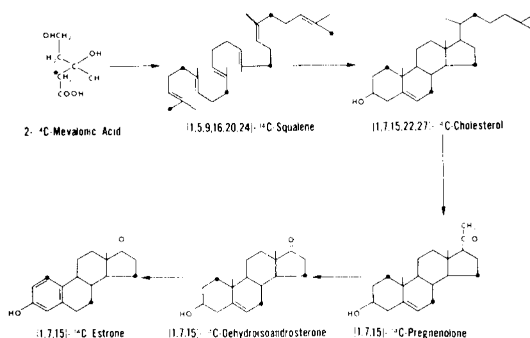


FIGURE 15. Predicted labeling of steroids formed by cyclization of squalene biosynthesized from 2-^{14}C-mevalonic acid. The black circles indicate the labeled positions.

the same as in the equivalent positions of the cholesterol molecule.[121] Therefore, if both estrone and equilin were formed by the same pathway and hence utilized the same precursors, they would both be radioactive. ^{14}C-Squalene is transformed to the classical estrogens but not to equilin or equilenin, indicating that ring B unsaturated estrogens are formed by a route which is independent of squalene and cholesterol. As expected, the pregnane derivatives 3β-hydroxy-5α-pregnan-20-one,5α-pregnane-3β, 20β-diol and 5α-pregnane-3β,20α-diol isolated contained ^{14}C-labeled material (Table 9).

In the next experiment, the precursor role of 2-^{14}C-DL mevalonic acid was investigated.[122] A total of 8.3×10^9 dpm of 2-^{14}C-mevalonic acid (specific activity 4.7 mCi/mmol) and 20×10^6 dpm ^{3}H-dehydroepiandrosterone (specific activity 10.4 Ci/mmol) was injected into the umbilical circulation of a horse fetus. Approximately 12% of ^{14}C-labeled material was recovered in the urine in 3 days. The phenolic fraction contained 14.0×10^6 dpm of ^{3}H and 32.0×10^6 dpm of ^{14}C, while the neutral fraction contained 4.7×10^6 dpm of ^{3}H and 69.4×10^6 dpm of ^{14}C.

Estrone, equilin, equilenin, and 17α-estradiol were isolated from the phenolic fraction and their radiochemical purity was established. The classical estrogens contained both ^{3}H and ^{14}C (Table 10), while the ring B unsaturated estrogens contained only ^{14}C. These results indicate that the route of biosynthesis of both the classical estrogens and the ring B unsaturated estrogens is the same up to the stage of mevalonic acid. Thus, the bifurcation in the classical pathway of steroid biosynthesis occurs at a point after the formation of mevalonic acid and prior to the formation of squalene.

It is interesting to note that the ^{14}C-specific activity of equilin was approximately 30 times lower than that of estrone (Table 10) when mevalonic acid is the precursor, while it is only 1.5 to 2 times lower when acetate is the precursor.[84,115,116] The full meaning of this difference in specific activity is not apparent at the present time. It is also interesting to note that the ^{14}C-specific activity of estrone and the three neutral steroids (Table 10) were not significantly different, again suggesting that they have a common biosynthetic pathway involving similar precursors.

4. Metabolism of 1-^{14}C-Isopentenyl Pyrophosphate and (4,8,12)-^{14}C-Farnesyl Pyrophosphate Injected into the Fetus

To further delineate the alternate pathway and to determine whether the bifurcation occurs immediately after the formation of mevalonic acid or just prior to the formation of squalene, the precursor role of isopentenyl pyrophosphate and farnesyl pyrophosphate were investigated.

In the first experiment 2.0×10^9 dpm of 1-^{14}C-isopentenyl pyrophosphate (specific activity 61 mCi/mmol) and 33.0×10^6 dpm of 7-^{3}H-dehydroepiandrosterone (specific

TABLE 10

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{14}C-Mevalonic Acid and ^{3}H-Dehydroepiandrosterone into the Umbilical Circulation of a Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	^{14}C	Weight (mg)
Equilin	0	830	49.0
Estrone	29,700	22,700	55.5
Equilenin[a]	0	890	1.5
17α-Estradiol[a]	19,000	10,000	5.0
3β-Hydroxy-5α-pregnan-20-one	0	29,000	100.0
5α-Pregnane-3β,20β-diol	0	25,100	159.0
5α-Pregnane-3β,20α-diol	0	26,200	20.0

[a] Due to small amounts isolated, these values are approximate and were calculated from the final specific activity and the carrier added.

activity 10.4 Ci/mmol) were injected into the horse fetus intramuscularly through the uterine wall,[123] 10% of ^{14}C was recovered in the maternal urine in 4 days. The phenolic fraction contained 9.7×10^{6} dpm ^{3}H and 4.2×10^{6} dpm ^{14}C, and the neutral fraction contained 1.4×10^{6} dpm ^{3}H and 2.6×10^{6} dpm ^{14}C. Estrone, equilin, 17α-estradiol, and equilenin were isolated and their radiochemical purity established. Since all steroids isolated contained ^{14}C (Table 11), the route of biosynthesis of both the ring B unsaturated and the classical estrogens is the same up to the stage of isopentenyl pyrophosphate, and the bifurcation in the pathway occurs at a point after the formation of isopentenyl pyrophosphate.

Bhavnani and Woolever[124] synthesized (4,8,12)-^{14}C-farnesyl pyrophosphate (specific activity 15 mCi/mmol) from 2-^{14}C-mevalonic acid and injected 2.2×10^{9} dpm of it and 30×10^{6} dpm of 7-^{3}H-dehydroepiandrosterone (specific activity 10.4 Ci/mmol) intramuscularly into a horse fetus.

Both the classical estrogens and the ring B unsaturated estrogens contained the ^{14}C-labeled material (Table 12), indicating that, like acetate, mevalonate, and isopentenyl pyrophosphate, farnesyl pyrophosphate is also transformed to both types of estrogens.

Hence, the bifurcation in the classical pathway occurs at a point after the formation of farnesyl pyrophosphate and before the formation of squalene (Figure 16). Since both classes of estrogens were obtained with farnesyl pyrophosphate, it is quite likely that both 3,3-dimethylallyl pyrophosphate and geranyl pyrophosphate would also be transformed to the ring B unsaturated estrogens. Therefore, only the precursor role of presqualene pyrophosphate in the formation of ring B unsaturated estrogens remains to be established.

The ^{14}C specific activity of equilin in both experiments (Tables 11 and 12) is again lower than that of estrone and 17α-estradiol, suggesting that factors other than the precursors are involved in the differences between the formation of ring B unsaturated estrogens and the classical estrogens.

We have shown the formation of ring B unsaturated estrogens when acetate, mevalonate, isopentenyl pyrophosphate, and farnesyl pyrophosphate were used as precursors. These results are summarized in Figure 16. The biosynthetic pathway of ring B unsaturated estrogens and the classical pathway of steroidogenesis are common until

TABLE 11

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{14}C-Isopentenyl Pyrophosphate and ^{3}H-Dehydroepiandrosterone into a Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	^{14}C	Weight (mg)
Equilin	0	270	400
Estrone	490	380	535
Equilenin	0	230	10
17α-Estradiol	510	400	12
3β-Hydroxy-5α-pregnan-20-one	0	640	195
5α-Pregnane-3β,20β-diol	0	570	114
5α-Pregnane-3β,20α-diol	0	600	29

TABLE 12

Endogenous Specific Activity and Weight of Steroids Isolated from Maternal Urine Following the Injection of ^{14}C-Farnesyl Pyrophosphate and ^{3}H-Dehydroepiandrosterone into the Horse Fetus

Steroid isolated	Specific activity (dpm/μmol) ^{3}H	^{14}C	Weight (mg)
Estrone	13,660	1,970	166.0
Equilin	0	240	458.0
17α-Estradiol	14,890	1,810	16.0
17α-Dihydroequilin	0	240	52.0
3β-Hydroxy-5α-pregnan-20-one	0	2,900	216.0
5α-Pregnane-3β,20β-diol	0	2,170	634.0
5α-Pregnane-3β,20α-diol	0	2,660	86.0

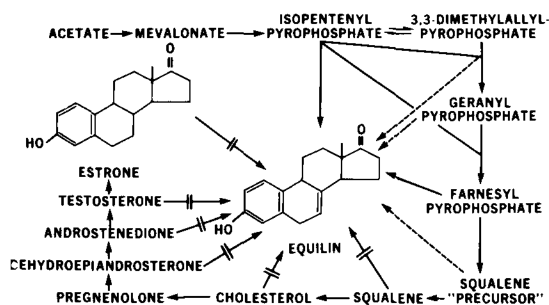


FIGURE 16. Pathways of estrogen biosynthesis in the pregnant mare.

the bifurcation occurs between farnesyl pyrophosphate and the formation of the carbon-30 hydrocarbon squalene. Whether or not presqualene pyrophosphate is an intermediate in the formation of equilin remains to be established. This potential precursor,

which is formed by condensation of two molecules of farnesyl pyrophosphate, has now been established as a precursor of squalene. It is quite possible that other intermediates may be involved in the biosynthesis of ring B unsaturated estrogens. It is conceivable that, during pregnancy in the horse, a sequence of reactions which usually forms squalene and cholesterol from farnesyl pyrophosphate undergoes a modification so that part of the intermediates serve as precursors for the ring B unsaturated estrogens equilin and equilenin, while the other part procedes through squalene and cholesterol to estrone.

5. Theoretical Discussion on Alternate Pathways of Steroidogenesis

If presqualene pyrophosphate can form ring B unsaturated estrogens, a search for new intermediates between presqualene alcohol and squalene is indicated. If presqualene pyrophosphate does not serve as a precursor for equilin and equilenin, then farnesyl pyrophosphate must give rise to presequalene pyrophosphate and to other intermediates such as the carbon-20 or carbon-25 pyrophosphates geranylgeranyl pyrophosphate or geranyl-farnesyl pyrophosphate. Recently, the theoretical possibility of steroidogenesis through a C_{25} sesterterpene (five isoprene units) has been suggested.[90-93] One can speculate that a C_{25} hydrocarbon on cyclization gives rise to a compound analogous to lanosterol but with five carbon atoms less on the side chain which, after demethylation and double bond migration, would give a compound analogous to cholesterol but with five carbon atoms less on the side chain, i.e., 23,24-dinor-5-cholen-3β-ol (Figure 17). Support for this hypothesis is based on the demonstration that 23,24-dinor-5-cholen-3β-ol can be transformed to 23,24-dinor-5-cholene-3β-20-diol (Figure 10) and the latter to cortisol by adrenal preparations.[90,92] Furthermore, it has been shown that these 23,24-dinor-cholene derivatives can be formed from acetate and not from cholesterol.[91]

It is also interesting to note that the analogue of lanosterol in this alternate pathway of steroidogenesis would be 23,24,25,26,27-pentanor-8-lanosten-3β-ol i.e., lanosterol with five carbons less in the side chain (Figure 17). The formation of this sterol has been shown in liver preparations incubated with synthetic substrates.[125,126] For both cyclization and the ensuing methyl hydrogen migration to take place, the basic system 2,3-oxide of C-25 hydrocarbon must be present.[125]

The C_{25} hydrocarbon could be formed by head to tail condensation of farnesyl pyrophosphate with isopentenyl pyrophosphate to give rise to geranylgeranyl pyrophosphate (C_{20}, four isoprene units); further condensation with isopentenyl pyrophosphate yields the C_{25}-pyrophosphate. The C_{25}-hydrocarbon can also be formed by a tail to tail condensation of farnesyl pyrophosphate with geranyl pyrophosphate (Figure 18)

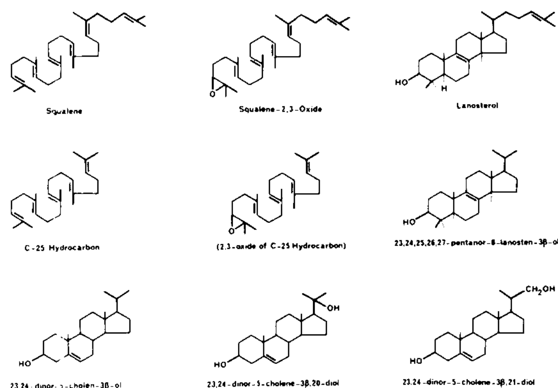


FIGURE 17. Structures of some new intermediates involved in biosynthesis of steroid hormones.

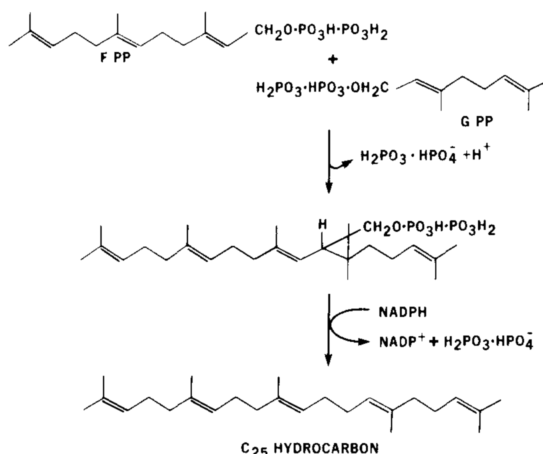


FIGURE 18. One possible mechanism for the formation of a C_{25}-hydrocarbon.

in a manner analogous to the condensation of two molecules of farnesyl pyrophosphate to give rise to squalene (Figure 5). The C_{25}-hydrocarbon formed can undergo enzymic cyclization as depicted in Figure 19. Such a transformation has been demonstrated by Anderson et al,[126] who reported that the absence of an entire isoprenoid unit (C_5) from the nonoxidized terminus of squalene oxide does not prevent normal biochemical cyclization.

The possibility of further lengthening the C_{15} pyrophosphate (farnesyl pyrophosphate) by a C_5 unit has been shown in the biosynthesis of carotenoids. The conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate (C_{20}, four isoprene units) by an enzyme system from bakers' yeast has been reported. Similarly, Kandutsch et al.[127] have purified geranylgeranyl pyrophosphate synthetase from *Micrococcus lysodeikticus*. This enzyme catalyzes the synthesis of geranylgeranol from isopentenyl pyrophosphate and either dimethylallyl, geranyl, or farnesyl pyrophosphate. The most rapid of the three reactions is the conversion of farnesyl pyrophosphate to the C_{20} pyrophosphate. The formation of terpene pyrophosphates containing up to 20 carbon atoms has also been shown with pig liver enzyme preparations. The presence of enzymes capable of producing still longer chain terpene pyrophosphates has also been described. The products formed by this system contained seven to ten isoprene units with a predominance of the C_{35} and C_{40} alcohols.[128] It is quite possible that similar allyl pyrophosphates, i.e., other than farnesyl pyrophosphate, may be involved in the formation of ring B unsaturated estrogens and other steroid hormones by yet undetermined mechanisms.

IV. BIOLOGIC ACTIVITY OF RING B UNSATURATED ESTROGENS

While the primary function of estrogens in the female mammal is the stimulation of estrous behavior and the growth and maturation of the reproductive system, they influence many other body systems and functions as well. Included among these are collagen; the skeletal system; the clotting mechanism; the concentration of protein, glucose, cholesterol, triglycerides, and electrolytes in plasma; the vascular and the nervous system; and the synthesis and release of pituitary gonadotropins. Some responses, including the plasma changes, reflect alterations in function of the liver or other organs and can be reproduced in various species. Others, such as the flushing and sweating associated with estrogen deficiency during the menopause in the human female, appear to be unique to individual species.

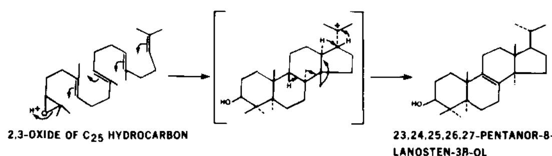


FIGURE 19. Cyclization of 2,3-oxide of C_{25}-hydrocarbon to 23,24,25,26,27-pentanor-8-lanosten-3β-ol.

Specific ring B unsaturated estrogens, like other estrogenic hormones, are known to stimulate the tissues of the reproductive system. In addition, they have been shown to modify the frequency and the severity of the flushes and sweats associated with estrogen withdrawal in the human female and to lower the concentration of gonadotropins in the serum and urine. However, their effect on other systems in the human is less clear and the available data primarily are derived from clinical studies using conjugated equine estrogen preparations, usually Premarin®. Obtained from the unhydrolyzed urine of pregnant mares, this product is a mixture of the classical estrogens (represented by the sodium sulfate ester of estrone (approximately 45%)) and the ring B unsaturated estrogens (represented by the sodium sulfate ester of equilin (approximately 25%)), plus lesser amounts of other estrogens of both classes (Table 13).[129,130] A similar product, known as "esterified estrogens," contains somewhat different proportions of the same compounds.[131] Highly soluble in water, the conjugated equine estrogens are easily absorbed from the gut in effective concentrations and have been used extensively as replacement therapy for estrogen-deficient women. Since the biological responses these compounds elicit are the sum of the effects of the various constituents, it is not possible to arrive at a reliable estimate of the potency of any specific ring B unsaturated compound from data derived from studies using these mixtures.

However, comparisons of many of the individual estrogens have been carried out in both animals and in humans. These studies indicate that, depending upon the type of preparation and route of administration, some of the ring B unsaturated estrogens are at least as potent as the classical estrogens, estrone, and estradiol.

V. BIOASSAYS

A. General

Biologic responses to specific stimuli will vary according to the species, breed and pretreatment of the test animals, caging and feeding conditions, and other, more subtle factors. The technique used to obtain tissues or vaginal smears as well as the method of preparation and the route of administration of the test compound also can result in major quantitative differences in the response. When the hormone is given by the oral, subcutaneous, intravenous, or intramuscular routes, the reaction may be due to a metabolite of the test substance rather than to the hormone in its original form.[133] Even when the test substance is applied directly to the tissue in which the response is to be measured, as in the vaginal application of an estrogen, the vehicle in which the hormone is carried can alter the quantitative effect.[133,134]

Careful control of these variables increases the reliability of potency comparisons made between substances in a single experimental protocol. However, it is difficult to compare the data from any one experiment to those derived from others, particularly when different end points are used, e.g., vaginal cornification vs. uterine weight. Even in animals bioassays, conflicting results frequently occur in spite of careful control of these variables. Studies in the human are much less precise because of the difficulties associated with obtaining standardized subjects and in controlling the living conditions during a trial.

TABLE 13

Components of Conjugated Equine Estrogen Preparations

Sulfate esters of:

Ring B saturated compounds[a]
- Estrone (45%)
- 17α-Estradiol
- 17β-Estradiol

Ring B unsaturated compounds
- Equilin (25%)
- 17α-Dihydroequilin
- 17β-Dihydroequilin
- Equilenin
- 17α-Dihydroequilenin
- 17β-Dihydroequilenin

[a] All compounds present in the form of sulfate esters.

B. Animal Bioassay Studies

Estrogen bioassay systems commonly measure the ability of the test compounds as compared to that of a standard compound to promote growth in the tissues of the reproductive tract. Cell division and multiplication most often are assessed only indirectly by changes in organ weight or by the number of mature cells in the vaginal smear.

1. Allen-Doisy Test

The end point of this assay as described originally[134] is the appearance of nucleated or cornified epithelial cells in the vaginal smears taken from castrated, estrogen-treated female mice.

Table 14 illustrates the difficulties in interpreting and comparing data from this type of assay when the several variables are not kept constant.[135] Here, two different forms of the same hormone (sodium estrone sulfate and estrone) were given by two different routes (oral and subcutaneous) to two species of animals (mouse and rat) pretreated in two different ways (intact and ovariectomized).

Estrone sulfate is more potent than estrone when given orally to the ovariectomized rat. It is less potent in the ovariectomized mouse and of about equal potency in the intact immature rat, when given in the same manner. By subcutaneous route, the same quantitative effect can be elicited with less of both hormones in both species if the adult animals are castrated. However, in the immature rat, the sulfate ester appears to be equally potent when administered by either route, while the unconjugated estrone is more potent if given by the subcutaneous path. The smaller effective doses required in the mouse as compared to the rat may be related to the size of the two animals.

Table 15 compares several estrogens in the Allen-Doisy test carried out in primed ovariectomized adult Wistar rats.[135] The figures indicate the dose level at which 50% of the animals had positive vaginal smears (RD_{50} — the dose producing a 50% positive response). The subcutaneous route is most effective for each of the compounds tested.

Equilin and equilin sulfate exhibit several times the potency of equilenin and its sulfate. When given orally, equilin and estrone have about equal activity, but the sulfate of equilin is marginally more potent than estrone sulfate. When given by the subcutaneous route, estrone exhibits somewhat greater activity than does equilin in both the conjugated and the unconjugated forms. The potency of 17β-estradiol is about equal to that of estrone and equilin. In contrast to the effect of conjugation on the potency of equilin and estrone given by the oral route, 17β-estradiol sulfate is much less potent than is its parent hormone.

TABLE 14

Comparative Activity in the Allen-Doisy Test (RD_{50} as micrograms estrone)[a]

Method	Administration	Sodium Estrone Sulfate	Estrone
Adult ovariectomised rats	Oral	85—110	158—249
	Subcutaneous	3—4	1.5
Adult ovariectomised mice	Oral	3—5	0.3—0.7
	Subcutaneous	0.6—0.7	0.06—0.08
Intact immature rats	Oral	7—8	6.3, 6.4
	Subcutaneous	6.2, 9.7	2.7—3.0

[a] The dose producing a 50% positive response.

TABLE 15

Comparative Activity of Estrogens and Estrogen Sulfates in the Allen-Doisy Assay (RD_{50} as micrograms estrogen total dose)[a]

Compound	Oral	Subcutaneous
Estradiol	170,198	0.1
Sodium 17β-estradiol sulfate	1040	—
Estrone	158—249	0.7—1.6
Sodium estrone sulfate	85—110	3—4
Equilin	200—210	1.4
Sodium equilin sulfate	78, 87	7.9
Equilenin	1000	70
Sodium equilenin sulfate	0840—117	>105

[a] The dose producing a 50% positive response.

2. *Uterotrophic Assay*

The end point of this assay is an increase in uterine weight. This reflects accumulation of interstitial fluid and the greater vascularity of the organ as well as an increase in the number and size of cells in response to estrogen.

Information on the relative potency of the 17-reduced forms of estrone and equilin are provided by Dorfman and Dorfman,[136] who fed the test hormones to immature albino rats by stomach tube over a period of 4 days. The results are expressed as the "uterine ratio" (100 × uterine weight in milligrams per gram of body weight).

Table 16 presents data selected for similarity of the size of the doses of the compounds being tested.[136] The sulfates of estrone and of equilin have similar uterine ratios in this assay. 17 β-reduction increased the uterotrophic activity of both, but the increase in the activity of equilin is considerably greater than that of estrone.

A more precise comparison was carried out by the same authors (Table 17).[136] The three unknown compounds were tested against sodium estrone sulfate in separate assays in each of which the response to estrone was assigned an arbitrary value of 100%. Sodium equilin sulfate and sodium estrone sulfate again are shown to be of approximately equal potency. 17β-Reduction brought about a 2.6 times increase in the activity of estrone sulfate but an 8.0 times increase in that of equilin sulfate. It may be significant that the metabolic pathways in the human are most likely to produce 17β-dihydroequilin sulfate instead of the 17α-dihydroequilin sulfate that is dominant in the pregnant mare.

TABLE 16

Rat Uterine Response to Orally Administered Sodium Estrogen Sulfates

Preparation	Total dose (μg)	Mean uterine ratio ± standard error
0	0	54 ± 3.7
0	0	56 ± 1.6
Sodium estrone sulfate[a]	2.5	77 ± 5.7
	5.0	108 ± 9.4
	10.0	141 ± 7.8
Sodium estrone sulfate[b]	4.0	67 ± 2.6
	8.0	104 ± 8.9
Sodium equilin sulfate	4.0	75 ± 2.6
	8.0	108 ± 5.2
Sodium 17β-estradiol sulfate	2.0	86 ± 8.0
	4.0	103 ± 5.4
Sodium 17β-dihydroequilin sulfate	2.0	151 ± 6.0
	4.0	191 ± 4.6
	8.0	196 ± 5.8

[a] Synthetic preparation.
[b] Prepared from impure estrone from pregnant mare urine.

TABLE 17

Rat Uterine Response to Orally Administered Sodium Estrogen Sulfates

Pair	Estrogens Assayed		Total dose (μg)	Potency ratio (%)
	Standard	Unknown		
1	Sodium estrone sulfate		4, 8	100
		Sodium 17β-estradiol sulfate	2, 4	261
2	Sodium estrone sulfate		4, 8	100
		Sodium equilin sulfate	4, 8, 16, 32	110
3	Sodium estrone sulfate		4, 8, 16, 32	100
		Sodium 17β-dihydroequilin sulfate	2, 4	882

3. Chick Oviduct Assay

This assay used changes in the weight of the reproductive system of chicks as a measure of estrogenic potency. The hormones have been administered subcutaneously[137] or mixed in with the food.[138] Although the food and hence the estrogen intake might be expected to vary when the chicks are allowed to eat at will, there is a remarkably small standard error in the results shown in Table 18.[138] A dose-related response is evident. In this assay, estradiol sulfate appears to be somewhat less effective than estrone sulfate. The conjugated equine estrogen mixture found in Premarin is more potent than either estrone, estrone sulfate, or estradiol sulfate at both the 40- and 80-mg dose level.

One can only speculate on the reasons for the apparently greater potency of this mixture of classical and ring B unsaturated estrogens as compared to the classical hormones alone. Equilin sulfate may act either in additive manner or in synergy with

TABLE 18

Chick Response to Orally Administered Estrogens

Preparation	Dosage as mg/kg food	Mean oviduct ratio ± standard error
0	0	16 ± 0.5; 16 ± 0.5; 14 ± 0.5
Estrone	20	24 ± 2.0
	40	40 ± 3.0
Sodium estrone sulfate (pure)	40	31 ± 4.0
	80	115 ± 11.0
	160	466 ± 90.0
Sodium estrone sulfate (crude)	40	39 ± 3.8
	80	142 ± 19.0
Sodium 17β-estradiol-3-sulfate	40	28 ± 4.0
	80	78 ± 13.0
	160	470 ± 129.0
Conjugated equine estrogens (Premarin®)	40	63 ± 8.0
	80	192 ± 15.0

estrone sulfate to account for the increase, or it may be that the augmentation is the result of a metabolic by-product of the parent hormone(s).[131]

Of the three bioassay systems that have provided these data, only the Allen-Doisy test directly examines the effect of the estrogens on cell growth. However, it has been established that the uterotrophic assay,[139] as well as the vaginal cornification response,[140] is associated with a marked increase in mitotic activity in the target organs. This growth reponse is similar to the activity seen in neoplastic tissues, but it does not lead to the conclusion that the estrogens are carcinogenic.

C. Human Studies

1. Specific Responses

Quantitative comparisons of estrogens in the human are usually carried out in amenorrheic, estrogen-deficient women. Some end points are similar to those used in the animal assays, e.g., vaginal cornification. Uterine responses estimate the stimulatory effect of estrogen on the endometrium either by histologic examination of tissue obtained at biopsy or by the appearance of uterine bleeding associated with regression of the endometrium after withdrawal of the estrogen stimulus (withdrawal bleeding). Like the animal assays, these end points which measure the growth-promoting ability of the estrogens are not to be confused with, or assumed to be synonymous with, any presumed carcinogenic effect. This has not yet been demonstrated.

The effect of estrogen on the hypothalamus or the pituitary is evaluated by measuring changes in the concentration of serum or urinary gonadotropins. A more subjective end point which is much more difficult to determine is suppression of the hot flushes and sweats that are associated with a decline in ovarian estrogen production during the climacteric. Inconsistencies in the description of the symptoms and the ability of certain nonestrogenic substances to control the symptoms in some women contribute to the confusion surrounding the results of these studies.

Specific ring B unsaturated estrogens have been used in only a few clinical trials, the results of which indicate that they are potent estrogens capable of eliciting responses that are qualitatively the same as those that follow administration of the classic hormones to humans. Equilin in doses from 0.5 to 2.mg/day was used in one of the earliest studies.[141] The vaginal epithelium responded readily at the lower dose level, the concentration of urinary gonadotropin was decreased by the 1-mg dose, and atrophic endometrium became proliferative over a 3-week course of treatment using daily doses of 1 and 2 mg.

Sodium equilin sulfate has been compared to sodium estrone sulfate and conjugated equine estrogens by following changes in the concentration of urinary gonadotropins in oophorectomized women. Continuous suppression of gonadotropin secretion was maintained by doses of 0.6 to 1.2 mg, 1.2 to 2.5 mg, and 2.5 to 10 mg, respectively, suggesting that sodium equilin sulfate alone is more potent than either sodium estrone sulfate or the combination of the two found in the conjugated equine estrogens.[142]

Orally administered 17-dihydroequilin sulfate in doses of 0.3 to 0.4 mg/day was found to be as effective as 0.625 mg of a conjugated equine estrogen preparation for the control of flushing and sweating.[143] Uterine bleeding associated with proliferative and hyperplastic endometrial growth also occurred at this dose level. It is not clear whether the compound used was 17α-dihydroequilin sulfate or 17β-dihydroequilin sulfate, although techniques commonly used for synthesis would suggest that most likely it was the latter compound.

This assumption is supported by the results of a later study in which 17α-dihydroequilin sulfate was found to have very little estrogenic activity in humans.[144] Continuous suppression of urinary gonadotropins was used as the end point to develop potency ratios for several estrogens. The conjugated equine estrogen preparation was assigned an arbitrary value of one. Others were tested and their potency ratios were: diethylstilbestrol, 2.5; equilin-3-monosulfate, 2.0; estrone-3-monosulfate, 0.4; 17α-dihydroequilin-monosulfate, 0.12; 17α-estradiol-monosulfate, 0.06; and 17α-dihydroequilenin-monosulfate, 0.

These data again illustrate that equilin sulfate is a more potent estrogen than is estrone sulfate when given by the oral route. The 17α-reduced products of estrone, equilin, and equilenin had little or no biological activity in this study, suggesting that the compound used in the previous trial[143] was probably 17β-dihydroequilin sulfate.

The influence of the end point used in an assay, the chemical form in which the product is prepared for testing, and the route of administration are illustrated by the different relative potencies assigned by other authors.[145] When uterine bleeding was used as the end point of testing and an arbitrary value of one was assigned to diethylstilbestrol, the following potency ratios were obtained: conjugated equine estrogens, 0.49; equilin, 0.37; and estrone, 0.05. The free or unconjugated forms of equilin and estrone appear to be less potent than the combination of sulfate esters found in the conjugated equine estrogens.

In a dose of 0.3 mg, equilin sulfate was found to be equal to 0.625 mg of conjugated equine estrogen in suppression of vasomotor symptoms and stimulation of the vaginal epithelium in a more recent study.[146] This potency ratio of approximately 2:1 is similar to that found in the earlier studies.[142,144]

The data from these studies are very much in agreement with the findings from the earlier animal experiments

1. Equilin and 17β-dihydroequilin are potent estrogens.
2. Equilenin and 17α-dihydroequilenin have little activity.
3. The sulfate esters are more potent than the parent compounds when given by the oral route.
4. Equilin and equilin sulfate are more potent than estrone and estrone sulfate.
5. Equilin sulfate given alone is more potent than an equal weight of the conjugated equine estrogens.

It may be of some clinical importance that the preferred pathway of metabolism of equilin in the human would lead to 17β-dihydroequilin rather than 17α-dihydroequilin. We have seen that the 17β form is much more potent than its parent compound in the uterotrophic assay[135] and that it is an active estrogen in humans.[143] These facts suggest

that equilin sulfate and 17β-dihydroequilin sulfate may be responsible for much of the endometrial stimulation associated with clinical use of the conjugated equine estrogens.

2. Other Responses in the Human

The widespread use of oral contraceptives has focused attention on the biological effects of the specific estrogens commonly used in them. Conjugated equine estrogen preparations have been used for estrogen replacement therapy in older women for many years, but scant attention has been paid to the effect of the specific ring B unsaturated estrogens on functions other than those directly related to reproduction. Even their role in bone growth and metabolism (for which the mixed preparations have been used so extensively) has not been investigated.

The differences found between the effects of the classic and the ring B unsaturated estrogens on the reproductive system are quantitative rather than qualitative. Similarly, it is likely that these unique estrogens influence other systems and function in a manner similar to, but perhaps quantitatively different from, the classic and synthetic estrogenic hormones. Some insight into their effect may be gained by consideration of clinical studies using conjugated equine estrogen preparations. It must be remembered that the response to these products, which contain a mixture of the classic and the ring B unsaturated hormones, is the sum of the effect of all of the ingredients.

a. Hypothalamus and Pituitary

Using conjugated equine estrogen preparations, both positive and negative feedback responses can be elicited in the human. Small doses (1.25 mg) are effective in suppressing and maintaining suppression of gonadotropins in postmenopausal women,[149] while relatively large amounts (3.75 mg for 21 days) are required to suppress the mid-cycle L.H. peak of the ovulatory cycle.[147-149] A positive response, i.e., a release of gonadotropin, has been observed after larger doses (20 mg) were given intravenously both to women with normal menstrual cycles[150,151] and to men.[152]

b. Clotting Mechanism

The recorded facts are contradictory. A reported increase in platelet aggregation without changes in adhesiveness or electrophoretic mobility[153] has not been confirmed.[154,155] Thromboelastography studies also are equivocal, some showing no change,[154] and others showing a shift toward hypercoagulability.[156] Changes noted early in the treatment schedules may revert to pretreatment status as the therapy is continued.[154]

Large intravenous doses of conjugated equine estrogens given to control bleeding associated with open-heart surgery have been reported to reduce blood loss without altering the clotting mechanism.[157] Using a somewhat different dose schedule, other observers found no change in either blood loss or in the clotting mechanism.[158]

Early reports indicated that the conjugated equine estrogens controlled bleeding by producing a thickening of the basement membrane of the capillary.[159,160] A more recent study has not supported this finding.[161]

Greater practical importance may be attached to the reported increase in the incidence of thromboembolic disease among older women using the hormonal (contraceptive) preparations.[162] Venous thrombosis occurs in 68 of 100,000 contraceptive users but in only 18 of 100,000 women using 0.3 to 1.25 mg of conjugated equine estrogens. However, this incidence is still twice that which is found in women using neither drug.[163]

c. Metabolic Changes

The serum concentrations of proteins and lipids and the tolerance to glucose are

altered in pregnant women and in nonpregnant women using exogenous estrogens. The observed changes are similar to those that are thought to predispose to atherosclerosis. These observations are difficult to reconcile with the evidence that the incidence of ischemic heart disease, which also is associated with atherosclerosis, increases in postmenopausal, estrogen-deficient women.[164]

The metabolic responses to estrogen are seen to vary during the course of treatment and with the length of therapy.[165-167] Decreases in glucose tolerance observed after 1 to 3 months have been reported to return to normal as treatment was continued over 2 to 3 years.[165] Cyclic therapy with conjugated equine estrogen resulted in an improvement in some diabetics while normal subjects receiving the same medication developed "diabetic" tolerance curves.[167] This observation prompted the authors to suggest that the hormone is glucogenic rather than diabetogenic and that only those patients who already were prediabetic before receiving the hormone will develop gross changes in carbohydrate tolerance.

Conjugated equine estrogens, ethinyl estradiol, and menstranol were found to produce somewhat different metabolic effects. Women taking conjugated estrogens had a small increase in fasting blood sugar, but none of the three hormones caused any significant change in the concentration of plasma insulin or tolerance to glucose after 6 months of therapy. Mestranol and ethinyl estradiol, but not the conjugated equine estrogen preparations, were associated with an increased concentration of plasma growth hormone.[168]

Serum concentrations of cholesterol, triglycerides, β-lipoproteins, pre-β-lipoproteins, and chylomicrons were seen to fall at the end of 3 months of treatment only to return to normal when the treatment was continued for 9 months.[166] Other observers who have treated women over a 1- to 5-year period have recorded a significant fall in serum cholesterol and in total lipids without any change in phospholipids.[169]

d. Osteoporosis

The earlier and more rapid rate of bone loss which occurs in older women as compared to older men is attributed to the decline of ovarian function and associated estrogen deprivation.[170] Although women have been treated by estrogen replacement in an effort to prevent or reverse this change, the mechanism whereby estrogen modifies bone metabolism is not any clearer now than when Albright advocated this therapy in 1941.[171]

A recent study showed that the rate of loss of bone density in oophorectomized women was slowed from 2.7 to 0.4%/year by small doses of mestranol. This was accompanied by a fall in serum calcium, phosphate, and alkaline phosphatase as well as in urinary calcium and hydroxyproline. A simultaneous decrease in the reabsorption of phosphates resulted in hyperphosphaturia.[172]

Conjugated equine estrogens produced a similar fall in serum calcium, phosphorous, alkaline phosphatase, and in urinary calcium while serum immunoreactive parathyroid hormone was found to increase during the treatment period. A decrease in bone-resorbing surface during the early phases of therapy was offset by a decrease in bone-forming surfaces as the treatment was prolonged.[173]

e. Atherosclerosis

The reported increase in the incidence of pulmonary and cerebral thromboembolism and of myocardial infraction in women over 35 years who were using the oral contraceptives[174,175] must be considered in the light of earlier laboratory and clinical observations indicating that estrogens reduced mortality from coronary and cerebral vascular diseases by retarding atherosclerosis.[176,177]

Conjugated equine estrogens have been used in clinical trials carried out on men with known atherosclerosis. In one group chosen for previous history of myocardial

infarction, there was an increased incidence of recurrent nonfatal infarction while on therapy.[178] A second group chosen for previous cerebral atherosclerotic disease showed a slight decrease in the frequency of myocardial infarction while on treatment. There was no change in the recurrence rate of cerebral infarcts in the latter group, but there was an increase in the incidence of mesenteric thrombosis, pulmonary embolus, and heart failure.[179]

A recent study of postmenopausal women indicated that estrogen replacement does not alter the incidence of myocardial infarction.[180] These results offer indirect evidence that the atherosclerotic process is not altered significantly by estrogen therapy, while the effect of the contraceptive pills on the clotting mechanism is clearly documented.

f. Neoplasms of the Reproductive Organs

The relationship between estrogens and neoplasms of the reproductive tract has been of interest for many years. Identification of the estrogen receptors in endometrial and breast tissues has contributed to knowledge of the mechanism by which estrogen stimulates growth in these tissues.[181] Recent observations indicating that women who have hyperplasia or carcinoma of the endometrium convert androstenedione to estrone in large amounts[182,183] have stimulated epidemiologic studies on the relationship between the use of exogenous estrogens and carcinoma of the endometrium and breast.

The results are equivocal. Some authors conclude that there is no relationship[184] or only a slight increase[185] in the relative risk of cancer of the breast in postmenopausal women using conjugated equine estrogens. On the other hand, carcinoma of the endometrium is reported to show a much higher incidence in association with the use of exogenous estrogens.[186]

Epidemiologic studies seldom prove a cause and effect relationship, but these studies do indicate that both endogenous and exogenous estrogens may play some role in the pathogenesis of carcinoma in estrogen-responsive tissues. Atrophic endometrium can be stimulated to the proliferative and then to the hyperplastic state in 4 months or less under the influence of exogenous estrogens.[187] However, the transition from this phase to atypical hyperplasia or frank malignancy is not inevitable, and the endometrium may revert to a proliferative condition or even to atrophy after curettage in spite of continued exogenous estrogen stimulation.[188]

These findings indicate that, in itself, estrogen is not necessarily carcinogenic, but rather, that it is a growth-promoting hormone that may play a role in the development of carcinoma of the endometrium or of the breast under certain conditions related to:[189]

1. The dose
2. The chronicity of exposure
3. The age of the patient
4. The ethnic or genetic derivation of the patient
5. The potential presence of predisposing virus-like factors affecting the ultimate hormone response of the tissues

It is of some importance to the present topic that the estrogen most commonly referred to in studies on the relation between estrogen and carcinoma of the endometrium is a conjugated equine estrogen preparation. The largest part of the compound is estrone sulfate, which is the dominant endogenously produced estrogen in the plasma of postmenopausal women. The ring B unsaturated estrogens which it contains are at least equal to estrone in their ability to stimulate the endometrium.

It will be necessary to learn much more about the patterns of absorption, metabolism, and the mode of action of the ring B unsaturated compounds before the role of

this preparation can be appreciated. This is relevant to not only neoplasia of the reproductive tissues but also to the various metabolic changes associated with the use of conjugated estrogens.

D. Metabolism of Equilin in Man

Equilin sulfate is available for therapeutic use as a major component of both the intravenous and oral preparations of conjugated equine estrogens.[131] The chemical form in which this ring B unsaturated estrogen is absorbed from the gut and carried to its site of action is unknown. There are no published data describing its half-life, metabolism, or excretion in man.

We have begun a series of experiments to determine the fate of equilin and of equilin sulfate when they are injected intravenously into the human. They are discussed in the following sections.

1. Intravenous Equilin Sulfate

10 mg of a conjugated equine estrogen preparation (Premarin®) containing approximately 2.5 mg of equilin sulfate and 4.5 mg of estrone sulfate (Table 13) was given as an intravenous bolus to a postmenopausal woman. Venous blood samples were taken at 10-min intervals. Unconjugated (free) steroids were extracted from the plasma with ether, leaving the conjugated estrogens in the aqueous fraction. Free estrone and equilin were isolated by chromatography and quantified individually by radioimmunoassay.[190] Figure 20 shows the concentration of free estrone and free equilin in the plasma in the first 80 min after injection. The peak concentration of both estrogens appeared at 20 min, after which both hormones were cleared rapidly but at somewhat

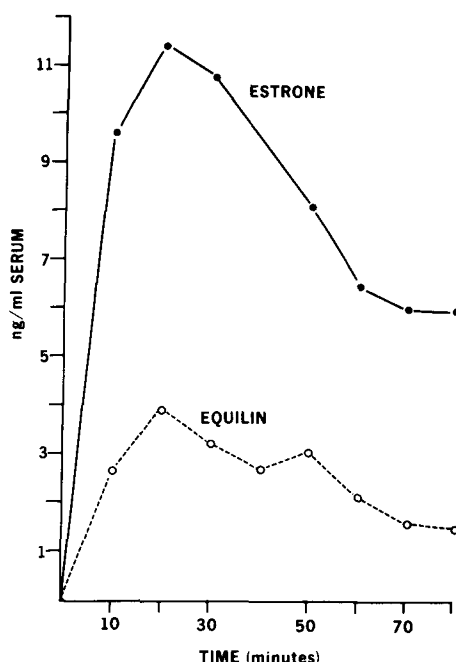


FIGURE 20. Concentration of free (unconjugated) estrone and equilin in the serum following a 10-mg intravenous bolus of conjugated equine estrogens (Premarine®). Equivalent to 5 mg of estrone sulfate and 2.5 mg of equilin sulfate.

different rates. The half-life of equilin was 43 min while that of estrone was 63 min in this experiment.

2. Intravenous ^{3}H-Equilin

6,9-^{3}H-Equilin was given to an adult male as an intravenous bolus (2.1 × 10^6 dpm, 3 μg) followed by an intravenous infusion (70.65 × 10^6 dpm, 100 μg) over 3 hr. Blood and urine samples were obtained as indicated in Figure 21. The concentration of radioactivity in the serum reached its maximum at the end of the infusion and remained stable for 1 hr, after which it fell in two phases. The first phase was relatively rapid. Subsequently, the decline was much slower. Small amounts of radioactive material were still present in the blood at the end of 72 hr.

The concentration of radioactivity in the urine also increased during the period of infusion but remained relatively stable for 5 hr after the infusion ended; 11 hr after the bolus had been given, 22.9% of the injected radioactivity had been recovered from the urine. After 24, 48, and 72 hr, 46.8, 65.1, and 72.3% of the radioactivity, respectively, had been put out in the urine. In the last 2 hr of the experiment, 225 ml of urine containing 71,775 cpm were collected.

In the pregnant mare, 6,9-^{3}H-equilin disappears from the plasma and appears in the urine much more rapidly after a single intravenous bolus (Figure 22). The disappearance of radioactivity from the serum and its appearance in the urine of the two species are compared in Figures 23 and 24. The half-life of the radioactivity in man, as represented in Figure 23, was calculated to be 5.2 hr from a second experiment in which 126 × 10^6 dpm of 6,9-^{3}H-equilin (2 Ci/mmol) was given as a single bolus. It is evident that the activity remains in the circulation of man for a much longer period than it does in the pregnant mare.

In the mare, 60% of the injected radioactivity was found in the urine at the end of 2 hr and 90% had been excreted after 24 hr.[192] In the human, 65.1% of the radioactivity appeared in the urine after 48 hr. At the end of 72 hr, 72.3% of the injected radioactivity was accounted for in the urine. The metabolites of equilin that appear in the urine of the two species also differ, both quantitatively and qualitatively. The free and the conjugated phenolic steroids were extracted and partitioned following the scheme shown in Figure 14.

The urine collected in the time interval 1 to 11 and 12 to 72, inclusive, (Figure 21) were combined and labeled pools "A" and "B", respectively; 10.4% of the radioactivity from the column eluate of pool A, and 0.57% of the activity in the eluate from pool B was found in the ether extract which contains free (unconjugated) steroids (Table 19). That which remains in the aqueous fraction is in the form of conjugates; 2.1

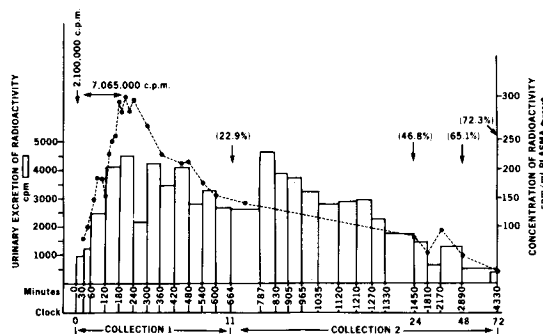


FIGURE 21. Concentration of radioactivity in the serum and the excretion of radioactivity in the urine of man after intravenous injection of ^{3}H-equilin.

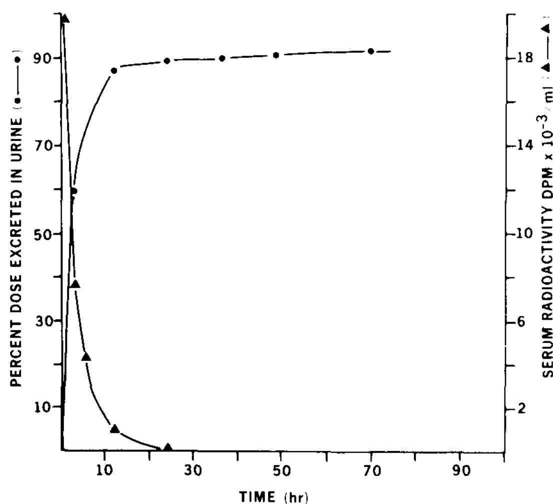


FIGURE 22. Concentration of radioactivity in the serum and the excretion of radioactivity in the urine of the pregnant mare after intravenous injection of ³H-equilin.

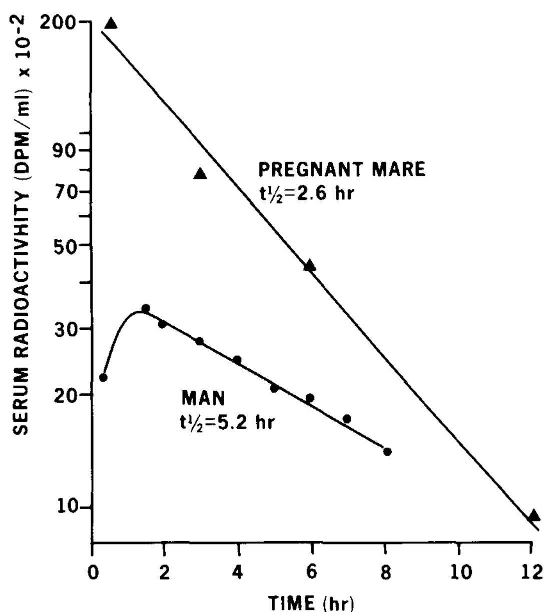


FIGURE 23. Concentration of radioactivity in the plasma of man and of a pregnant mare after intravenous injection of ³H-equilin.

and 7.0% of this radioactivity could be extracted from pools A and B, respectively, after solvolysis (sulfates), while 68.2% was recovered from pool A, and 76.4% from pool B, after incubation with β-glucuronidase (glucuronides).

These data indicate that small amounts of the free steroid were excreted early after injection but that most of the urinary metabolites appeared as glucuronides or as double conjugates (sulfo-glucuronides).

To assist in the identification of the metabolites represented by the radioactivity found in the urine, three males ingested large amounts of nonradioactive equilin and

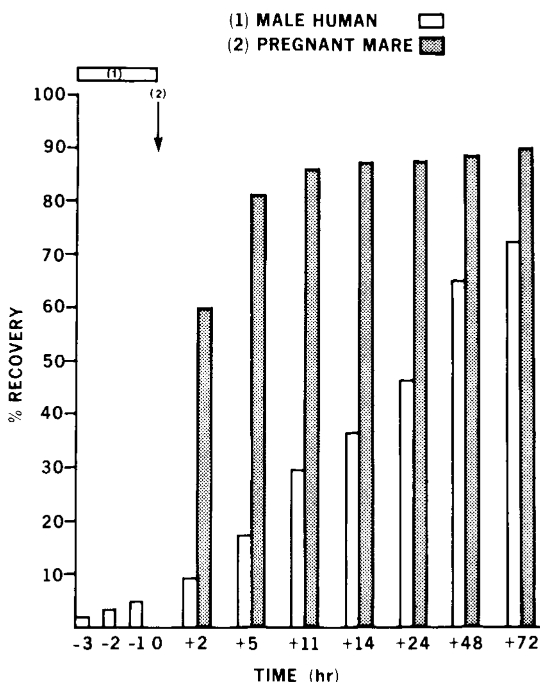


FIGURE 24. Appearance of radioactivity in the urine of man and a pregnant mare after intravenous injection of ^{3}H-equilin.

TABLE 19

Extraction and Partition of Radioactivity in the Urine after Intravenous Administration of 6,9-^{3}H-equilin in an Adult Male

	Urine pools		
	A	B	Total
Collection times (hr)	0—11	12—72	72
% Total dose recovered			
In urine	22.6[a]	54.1	76.7
From XAD column (methanol)	11.4	47.1	58.5
Fractionation of column eluate (= 100%)			
Ether — unconjugated/free	10.4	0.5	2.4
Total conjugates (as tetrahydrofuron extracts)	89.0	96.4	23.6
Aqueous	0.6	3.1	2.7
Sulfates	2.1	7.0	6.0
Glucuronides	68.2	76.4	74.8
Other conjugates	18.6	13.0	14.1

[a] Sum of fractions 1 to 12

Note: Injected: ^{3}H-Equilin. 42.5 × 10^6dpm

3-day combined urine collections were partitioned as described before (Figure 14). The phenolic fractions from this urine were added to those from the urine collected in the earlier experiment in which the 6,9-^{3}H-equilin was given as a single bolus. The combined extracts were purified on a 600-g Celite® partition column using the system heptane: benzene: methanol: water (10:5:8:2). The pattern of radioactivity found in

the column fractions is shown in Figure 25. A small amount of radioactive material was found in fractions 300 to 400 from which equilin and equilenin were isolated and identified. No radioactivity was found in the position of 17α-dihydroequilin, but a relatively large amount was found in the position of 17β-dihydroequilin and 17β-dihydroequilenin. The largest portion of the radioactive material was found in fractions 1500+, indicating that it consists of a very polar compound or group of compounds. In contrast, the pregnant mare does not metabolize equilin to materials more polar than dihydroequilin or dihydroequilenin (Figure 26). Other differences in the metabolites found in the mare include the presence of a large amount of nonreduced equilin and a significant amount of 17α-dihydroequilin. From the human urine, equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin have been isolated and their radiochemical purity established (Table 20).

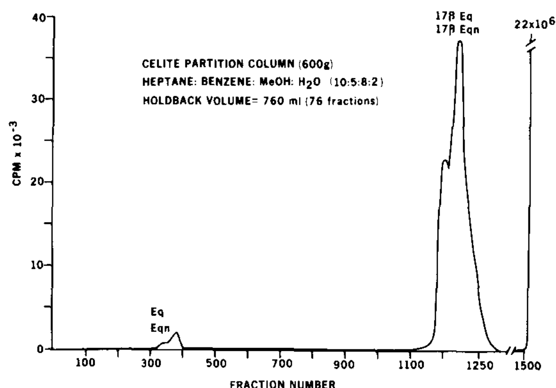


FIGURE 25. Purification of the urinary phenolic fraction by Celite® partition chromatography following intravenous administration of ^{3}H-equilin to a human male; Eq = equilin, Eqn = equilenin, 17β-Eq = 17β-dihydroequilin, 17β-Eqn = 17β-dihydroequilenin.

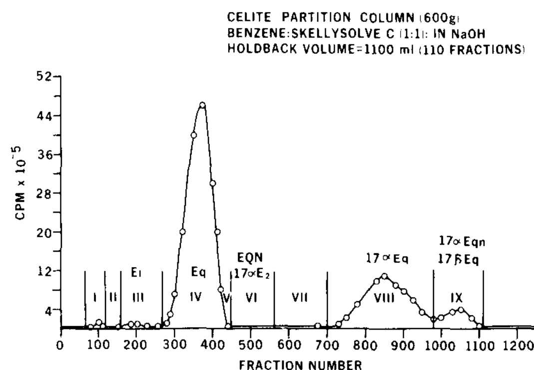


FIGURE 26. Purification of the urinary phenolic fraction by Celite® partition chromatography following the intravenous administration of ^{3}H-equilin to a pregnant mare; E_1 = estrone, Eq = equilin, Eqn = equilenin, 17α-F_2 = 17α-estradiol, 17α-E_2 = 17α-dihydroequilin, 17βEq = dihydroequilin, 17α-Eqn = 17α-dihydroequilenin.

TABLE 20

Proof of Radiochemical Purity of Metabolites Isolated from the Urine of Men after the Injection of 6,9-^{3}H-Equilin and the Ingestion of Unlabeled Equilin

	Specific Activity (dpm/mg)	
Crystallization	Equilenin	Equilenin-3-acetate
1	1670	1490
2	1680	1540
3	1590	1420
Calculated	1270	1370
	17β-Dihydroequilenin	17β-Dihydroequilenin-diacetate
1	2290	1710
2	2190	1720
3	2200	1660
Calculated	1750	1670
	Equilin	Equilin-3-acetate
1	980	770
2	960	770
3	850	800
Calculated	1010	730
	17β-Dihydroequilin	17β-Dihydroequilin-3-methyl-ether
1	2260	2200
2	2170	2210
3	2100	2180
Calculated	1790	2000

Known amounts of unlabeled equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin were added to the blood samples taken early in the course of the single bolus experiment in man (126×10^6 dpm of 6,9-^{3}H-equilin, 2 Ci/mmol). The serum was then fractionated into the "free," "sulfate," and "glucuronide" fractions. Only the free and sulfate fractions contained radioactive materials. The two extracts (free and sulfate fractions) of each sample were purified by paper chromatography, and radioactive areas corresponding in mobility to equilin and 17β-dihydroequilin were eluted. Radiochemical purity was established in some samples of equilin by isotope dilution techniques. The amount of radioactivity present in equilin and 17β-dihydroequilin corrected for procedural losses is given in Table 21.

The preliminary data indicate that the injected equilin is converted to equilin sulfate very rapidly. At 20 min, the ratio of free compound to the sulfate ester was approximately 1:2. At 40 min, the ratio was almost 1:6 and by 60 min, only a small amount of the free compound remained while the concentration of equilin sulfate continued to increase up to 90 min. 17β-Dihydroequilin appeared rapidly in the serum in both the free and sulfate forms. The free form disappeared quickly as the concentration of the sulfate increased (Table 21).

The radioactivity associated with the free steroid fraction of the serum sample had disappeared from the serum at the end of 3 hr. That which was present in the "sulfate fraction" increased rapidly, reaching a maximum concentration at 1.5 hr after which the radioactivity began to disappear at a much slower rate. The half-life of the total radioactivity during this phase is 5.2 hr (Figure 23), which is similar to the half-life of "total radioactivity" in the first phase of the experiment illustrated in Figure 21.

The data from these preliminary experiments must be enlarged and confirmed, but

they do suggest several tentative conclusions regarding the metabolism of equilin in the human:

1. Equilin sulfate can be rapidly metabolized to the free steroid found in the serum (Figure 20).
2. The free steroid is readily sulfated after intravenous injection (Table 21).
3. 17β reduction occurs soon (20 min) after intravenous injection of the free steroid but 17α reduction does not occur (Table 21 and Figure 25).
4. Equilin sulfate is the major metabolite found in the serum after the injection of the free equilin (Table 21).
5. Free equilin remains in the serum and is excreted into the urine for only a short period of time after injection (Tables 19 and 21).
6. Large amount of 17β-reduced compounds are found in the urine, but the bulk of the steroid is metabolized and excreted as more polar compounds (Figure 25).
7. Most of the urinary metabolites are excreted as glucuronides (Table 19).
8. The metabolites persist in the serum for days after a single injection (Figure 21).

These observations differ significantly from the metabolic pathways of equilin in the mare, the only species in which equilin is a "natural" hormone. The mare excretes the hormone very rapidly as equilin sulfate, and the major metabolite is the biologically weaker 17α-dihydroequilin sulfate rather than the more potent 17β-dihydroequilin sulfate that is found in the human. Both species can introduce a second double bond into the B ring to give rise to equilenin and its reduced metabolites.

There are a number of similarities between the metabolism of estrone and equilin in the human. Reduction to the β configuration at carbon-17 imparts an increased estrogenic potency to both compounds (Table 17). Estrone sulfate is the major form of the hormone in the serum.[193] Free estrone and free equilin have relatively short half-lives (Figure 23). Estrone and its metabolites are excreted in the urine primarily as glucuronides and large amounts of very polar compounds are found.[194,195]

The implications of these findings are of some importance in the choice of drugs for estrogen replacement therapy. Equilin and 17β-dihydroequilin can compete with estradiol for estrogen receptors in the uterus.[196] The clinical data also indicate that they are capable of entering the target cell nuclei and triggering the same growth mechanisms that are stimulated by the classical estrogens. There are no data related to their metabolic effects, but it is unlikely that they are inert with regard to calcium, carbohydrate, and lipid metabolism and other body systems in which both the classical, synthetic, and conjugated equine estrogens have been shown to be biologically active.

TABLE 21

Distribution of Equilin and 17β-Dihydroequilin in Human Serum Following Intravenous 6,9-^{3}H-Equilin (126 × 10^6 dpm)

Time after injection (min)	Equilin (dpm/mℓ)		17β-Dihydroequilin (dpm/mℓ)	
	Free	Sulfate	Free	Sulfate
20	2030	3920	1400	240
40	1180	6020	630	520
60	240	9400	560	600
90	190	10110	120	720

V. CONCLUDING REMARKS

We have discussed the evidence regarding the existence of an alternate pathway of steroidogenesis which is independent of cholesterol. The evidence regarding the formation of all steroid hormones by this route is not compelling and is based primarily on a few selected in vitro incubation studies. The triparanol studies (where desmosterol was shown to be converted to steroid hormones) indicate that the enzyme for the conversion of sterols to steroid hormones is not specific and that the animal body can utilize various sterol substrates (other than cholesterol) to produce steroid hormones. Whether these pathways of steroidogenesis contribute significant amounts of steroid hormones to the general pool in the body remains to be established.

The evidence regarding the formation of ring B unsaturated estrogens by pathways not involving cholesterol is more direct and conclusive. The ring B unsaturated estrogens, equilin, and equilenin are a unique group of hormones secreted by the pregnant mare. Their in vivo biosynthesis diverges from the classical pathway of steroidogenesis at a point before the formation of squalene and cholesterol. Though the equilin and equilenin are thought to be native only to the pregnant mare, it has been reported[197,198] that preparations of human feminizing testis and adrenal carcinoma are capable of producing equilenin, a metabolite of equilin. These observations point to the possibility that alternative pathways of steroid biosynthesis, as seen in normal pregnant mares, may be also occurring in humans under certain pathological conditions.

It is not yet known where these ring B unsaturated estrogens are being synthesized in vivo. It is unlikely that these hormones are biosynthesized by maternal tissues. Since the level of urinary equilin drops after intrauterine fetal death, it would appear that perhaps the fetus and the placenta are responsible for the production of these estrogens. There is some evidence[199] to indicate that fetal gonads may have some role in the production of estrogens in the pregnant mare. It is more likely that neutral precursors are produced by the fetal adrenal and liver and are then aromatized by the placenta in a manner similar to the production of estriol during human pregnancy.

The sulfate esters of equilin and equilenin along with estrone sulfate are widely prescribed for human use and collectively described as conjugated equine estrogens. In addition to the ring B unsaturated estrogens, these preparations contain a large amount of estrone and smaller amounts of its reduced forms. A third component probably is equol, a phenolic compound derived from alfalfa grass also found in the urine of other grass-eating mammals. This compound has no known estrongenic or other biologic activity.

Animal and human tests of the estrogenic potency of equilin, equilenin, and their 17-reduced derivatives center mainly about their ability to stimulate growth in the tissues of the female reproductive tract. Equilin and 17β-dihydroequilin are seen to be potent estrogens while equilenin and its 17β-reduced derivatives and the 17α-reduced derivatives of both have been shown to have little or no effect in the tests used. The metabolic effects of the ring B unsaturated compounds have not yet been investigated individually but only as components of the conjugated equine estrogen preparations.

Although few studies on the metabolism in the human have been carried out, it appears that equilin is handled in a manner similar to estrone, i.e., it is rapidly conjugated, reduced at the C-17 position to the corresponding 17β-hydroxy compounds, and excreted in the urine conjugated with glucuronic acid. Equilin and its metabolites are retained in the blood and tissues of the human much longer than in the pregnant mare.

Metabolic pathways in the human also are different from those in the mare, with the primary reduced form being the 17β-dihydroequilin as compared to the 17α-dihydroequilin in the mare. This metabolic pathway in the human may be important in light of the animal data indicating an eightfold increase in uterotrophic activity of the

17β-dihydroequilin as compared to equilin. The recent concern over the metabolic and endometrial effects of estrogens emphasizes the dearth of knowledge concerning these commonly used drugs. There is a marked need for further investigation of their synthesis, metabolism, and biologic activity.

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Chapter 2

FACTORS CONTROLLING THE BIOSYNTHESIS OF ALDOSTERONE

J.-G. Lehoux

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I. INTRODUCTION

The regulation of the biosynthesis of aldosterone in the adrenal gland is very complex due to both direct and indirect actions of the various control factors. This complexity is greater since the control factors concerned act at different sites, either outside the adrenal cell on the plasma membrane or inside the cell at the molecular level in enzymatic systems.

Prior to incorporation into cholesterol and aldosterone, acetate is metabolized by enzymes located in different subcellular compartments (e.g., cytosol, mitochondria, microsomes). Each enzymatic step and each transfer from one cellular compartment to another is a potential regulatory site of aldosterone formation.

It is well known that alterations in plasma potassium, sodium, angiotensin II, and ACTH levels will modify the rate of aldosterone synthesis. Recent reports indicate that prostaglandins, especially the E and A types, might also be involved in the synthesis regulation. Serotonin was shown to stimulate aldosterone secretion in vitro in the adrenal glands of many species, but evidence is lacking for a stimulating effect of this substance in vivo. Vasopressin, a neurohormone, might also have a role to play in the control of aldosterone secretion.

Interesting articles have been written on the control of aldosterone secretion by adrenals, and this chapter will discuss the mode and mechanism of action of these factors with emphasis on recent research in this field.

II. MINERALOCORTICOID ACTIVITY

The term "mineralocorticoid" has been applied to a class of steroids which have an effect on ion transport by epithelial cells that results in retention of sodium and loss of potassium. Aldosterone is the more potent mineralocorticoid among naturally occurring corticosteroids, followed in decreasing order of potency by 11-deoxycorticosterone, 18-hydroxydeoxycorticosterone, corticosterone, cortisol, and cortisone.

III. ANIMALS SYNTHESIZING ALDOSTERONE

A. Species

The biosynthesis of corticosteroids throughout the vertebrate kingdom has been recently reviewed.[1] Aldosterone is secreted by adrenocortical tissues of pratically all mammals investigated, including the whale. However, two known exceptions are the antarctic seal and the squirrel monkey; no plasma or adrenal aldosterone could be found nor in vitro synthesis from exogenous precursors by adrenal glands. Birds, reptiles, and amphibians secrete significant amounts of aldosterone.

Neither fish nor cyclostomata produce aldosterone. However, the lungfish is an exception to this rule as plasma aldosterone was found in high concentration. Cortisol was described as being responsible for sodium movements in the gills of Osteichthyes.[1a] 1α-Hydroxycorticosterone is the mineralocorticoid secreted by the Chondrichthyes' adrenocortical tissue; its activity was measured by bioassay on the toad bladder and found to be as high as that of aldosterone. In summary, the aldosterone-synthesizing capability of adrenocortical tissues appears to be almost completely limited to nonaquatic vertebrate species.

B. Tissues

Only adrenocortical cells seem to be capable of aldosterone synthesis. Avian and amphibian aldosterone-secreting cells are intimately intermixed with those of the medulla. In certain lizard species, a rudimentary differentiation appears between both med-

ullar and adrenocortical tissues. Mammalian adrenocortical cells are distinct and completely surround the medullar cells. The mammalian adrenal cortex is of mesodermal origin and composed of three concentric zones: the external zona glomerulosa is responsible for the hormones acting on electrolyte metabolism; the intermediary zona fasciculata secretes hormones acting on carbohydrate metabolism; and the inner zona reticularis is involved in the synthesis of adrenal androgens. Zona glomerulosa cells are smaller than those of zona fasciculata and reticularis and have a high nucleus cytoplasm ratio; according to Greep and Deane,[2] liposoluble steroid hormones are stocked in lipidic vacuoles prior to secretion. The weight of the adrenal in the adult animal is higher in females than males, this difference being due presumably to the adrenal-gonad interrelationship.[3]

In the adrenal gland, blood flows from arterioles that pass through the connective tissue capsule to the zonae glomerulosa and fasciculata. Blood then flows through the sinuses of the zona reticularis, enters the medulla, and leaves via the adrenal veins.[4] However, this does not exclude the small veins which pass through the capsule.

IV. ALDOSTERONE BIOSYNTHETIC PATHWAYS

A. Acetate to Cholesterol

Cholesterol is a good precursor for corticosteroids. It can originate from at least three different sources; adrenocortical cells can use their own stored cholesterol, extract it from plasma, or synthesize it *de novo* from acetate.[5] Adrenocortical tissues of man,[6] pig,[7] beef,[8] guinea pig,[9] rat,[5,10] hamster,[11] and bird[12] have been shown to possess the capability of synthesizing cholesterol from acetate. The significance of this pathway may vary depending on the species[13] and the needs of the animals under specifically defined conditions.

Following ACTH stimulation, in man, the conversion in vitro of radioactive acetate to adrenal cholesterol increased at a rate comparable to that observed for liver preparations.[6] Based on observations that radioactive acetate and other intermediaries such as mevalonic acid[14-16] and desmosterol[17] were incorporated into adrenal cholesterol or corticosteroids, it is believed that the pathway of adrenal cholesterol synthesis is the same as that for liver and yeast. The details of this biosynthetic pathway have been reviewed by Clayton.[18]

According to Rodwell et al.,[19] the HMG-CoA reductase necessary for the reduction of 3-hydroxy-3-methylglutaryl coenzyme *A* to form mevalonic acid in rat liver is the key regulatory enzyme of cholesterogenesis. This reductase is localized in the liver microsomal fraction and exhibits a diurnal rhythm accompanied by a parallel rhythm in the synthesis of sterol from acetate.[19-21] The regulation of cholesterol biosynthesis in the adrenal has not been as well investigated as that of the liver. However, the presence of HMG-CoA reductase activity was demonstrated in adrenocortical tissues of various vertebrate species.[22] The following adrenocortical HMG-CoA reductase activities were found (nmoles of mevalonic acid formed per milligram protein per 30 min): hamster microsomal fraction, 19.3; hamster mitochondrial fraction, 15.1; rabbit microsomal fraction, 2.8; rat microsomal fraction, 0.35; chicken microsomal fraction, 7.4; chicken mitochondrial fraction, 9.4; and frog microsomal fraction, 0.14.

The adrenal glands of different species have been broadly classified on the basis of total lipid concentration as "fatty" or "nonfatty" types.[23] According to Lloyd,[13] the nonfatty gland would synthesize more cholesterol than that of the fatty gland to compensate for differences in the quantity of stored cholesterol which serves as a precursor for corticosteroids. Hamster adrenals store very little cholesterol and have a high HMG-CoA reductase activity; by contrast, rat adrenals (which accumulate large amounts of cholesterol) have a very low adrenal HMG-CoA reductase activity. We

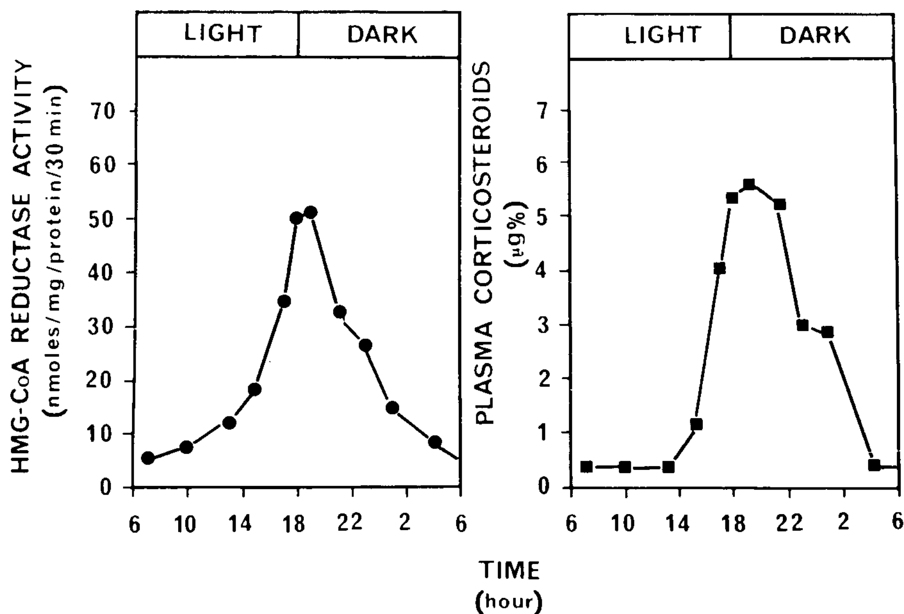


FIGURE 1. Diurnal variations of adrenal HMG-CoA reductase activity and plasma corticosteroid level in the male syrian hamster (From Lehoux, J.-G. and Preiss, B., unpublished data, 1977.)

have found[24] that the hamster adrenal HMG-CoA reductase, located in a heavy microsomal fraction, exhibits a diurnal rhythm with a maximum of activity at 19:00 hr under alternating 12-hr light-dark periods (light 6:00 to 18:00 hr). The maximum reductase activity was found to coincide with that of plasma corticosteroid concentrations as shown in Figure 1. The parallelism observed between these two cycles suggests a physiological link between the two phenomena.

Some evidence indicates that ACTH has an effect on both the synthesis and accumulation of cholesterol in the adrenal. ACTH was shown to accelerate the rate of incorporation of acetate into corticosteroid by bovine adrenal cortex.[8] In man, the experiments of Borkowski et al.[6] suggest that ACTH stimulates both the synthesis of adrenal cholesterol and the uptake of plasma cholesterol. Injection of ACTH to rats treated with aminoglutethimide, a drug that blocks the transformation of cholesterol to pregnenolone, resulted in an accumulation of adrenal cholesterol.[25] Robertson and Reddy[26] report that ACTH stimulated the incorporation of |1-^{14}C|acetate into mevalonate, cholesterol, and cortisol in calf adrenal cortex slices; when mevalonate was added to the incubation medium, a decrease of radioactivity incorporation into mevalonate resulted, suggesting that ACTH had an effect at a site preceding the formation of mevalonate. We have recently shown[24] that one injection of ACTH (2.5 Units per kilogram body weight) per day for 3 days to hamsters resulted in a three- to fourfold increase in the activity of adrenal microsomal HMG-CoA reductase activity. In addition, subcutaneous injections of aminoglutethimide (50 mg/kg body weight) twice a day for 3 consecutive days inhibited the HMG-CoA reductase activity by 40 to 60%. In rat adrenal, a 200-fold increase in the activity of microsomal HMG-CoA reductase was observed when plasma cholesterol was lowered by treating animals with 4-aminopyrazolopyrimidine.[27] These data are consistent with the possibility that HMG-CoA reductase activity is regulated by the size of the intracellular pool of cholesterol in the adrenal gland. Administration of aminoglutethimide to the hamster would lead to an increase in size of this pool, while ACTH would diminish it by stimulating the activity

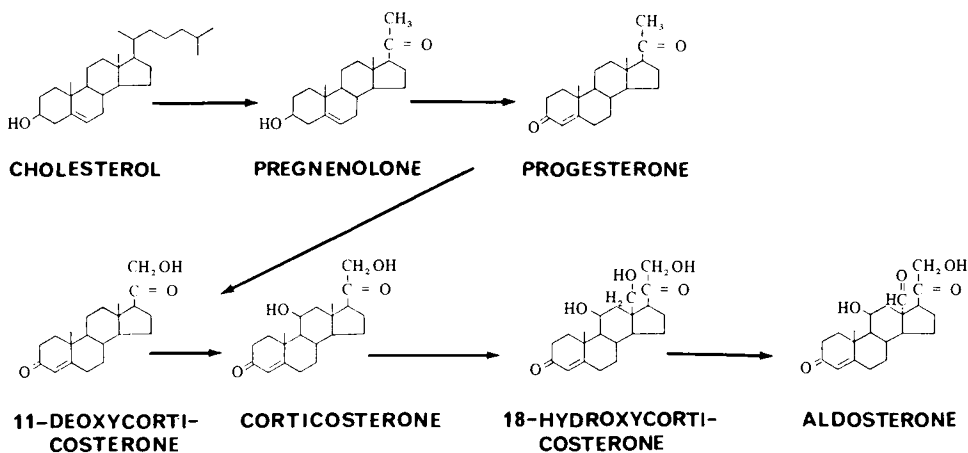


FIGURE 2. Pathway of aldosterone biosynthesis.

of the cholesterol side-chain cleavage. An ACTH effect on the regulation of the cholesterol pool in the isolated rat adrenal cells was also suggested by studies on cholesterol synthesis from mevalonic acid.[16]

B. Cholesterol to Aldosterone

The details of the cholesterol → aldosterone pathway have been recently reviewed by Sandor et al.[1] and Müller.[28] It is generally accepted that aldosterone originates from cholesterol mainly through the following classical pathway: cholesterol → pregnenolone → progesterone → deoxycorticosterone → corticosterone → 18-hydroxycorticosterone → aldosterone (Figure 2).

The cholesterol side-chain cleavage reaction takes place in mitochondria in the presence of molecular oxygen with NADPH as the reducing agent. The bioconversion of cholesterol to pregnenolone in adrenocortical tissue is regarded by many workers as a control point for corticosteroid biosynthesis. Accordingly, many efforts have been made to elucidate the details of the mechanism of this transformation, and a number of controversial hypotheses have been proposed. The classical approach depicts the side-chain cleavage as proceeding through a sequential, mixed-function oxidase with 20α- and 22R-hydroxylations of cholesterol to form 20α, 22R-dihydroxycholesterol via either the 20α- or 22R-hydroxycholesterol, with scission of the C_{20}-C_{22} bond of the 20α, 22R-glycol by a separate lyase yielding pregnenolone and isocaproicaldehyde.[29] Other intermediates leading to the 20α, 22R-glycol which have been proposed include the 20-hydroperoxycholesterol[30] and the 20, 22-epoxycholesterol.[31] However, the hydroperoxide route has been precluded by ^{18}O studies[32] whereas putative dehydro- and epoxy-intermediates are ruled out by metabolic studies on synthetic substrates.[33,34] Difficulties in detecting any intermediates at all during cholesterol incubations suggest that side-chain cleavage involves a concerted type of reaction from which only pregnenolone is released in the medium.[29,35] Consistent with this concept is the postulate that the true intermediates are not stable products, but rather, that they are transient, reactive radical, or ionic species.[36] However, recent kinetic studies by Burstein and Gut[37] on the early formation of intermediates during adrenocortical incubations with cholesterol strongly suggest that the sequence 22R-OH → 20α, 22R-diOH → pregnenolone is the basic route by which side-chain cleavage in the adrenal cortex takes place. These observations are in agreement with the scheme for side-chain cleavage recently proposed by van Lier et al.[38,39] Their system provides a model in which the epimeric 20-hydroperoxycholesterols serve both as substrate and as source of the "activated"

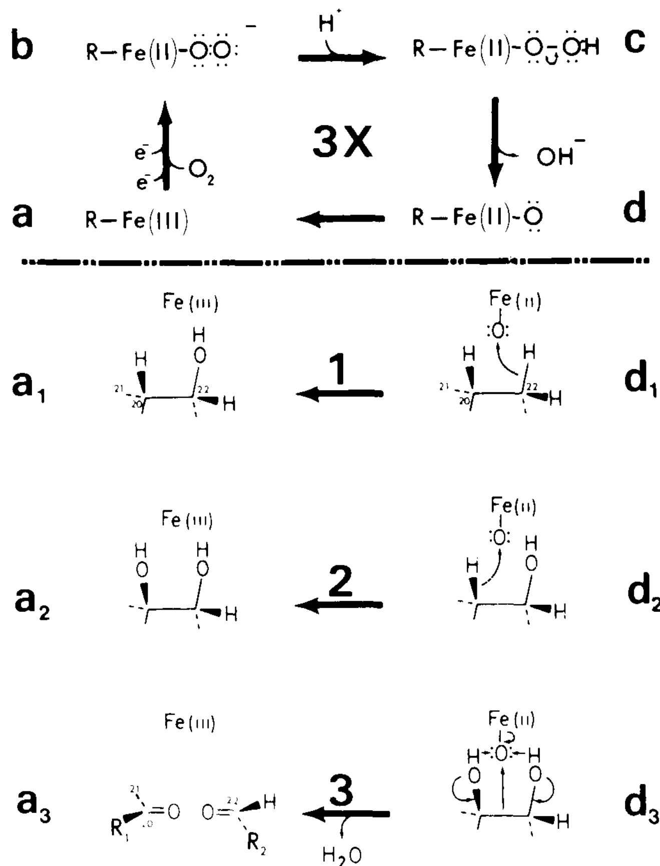


FIGURE 3. Proposed model for side-chain cleavage of cholesterol through three consecutive *in situ* attacks of a ferryl ion complex (d) on the C_{22}-H and C_{20}-C_{22} bonds of enzyme-bound cholesterol. (From van Lier, J. E. and Rousseau, J., *FEBS Lett.*, 70, 26, 1976. With permission.)

oxygen, resulting in stereospecific hydroperoxide-glycol rearrangements. Based on such findings, they propose that the side-chain cleavage of cholesterol involves three consecutive *in situ* oxidations through a ferryl-atomic oxygen complex of P-450 with pregnenolone as the final product (Figure 3). Using purified enzyme preparations, Morisaki et al.,[40] also obtained evidence suggesting that the three steps involve a single species of cytochrome P-450.

Pregnenolone travels from the mitochondria to the microsomes in which the enzymes necessary for its conversion to progesterone are located.[41] The oxidation of the hydroxyl group to a ketone at position 3 takes place in the presence of NAD^+ and 3β-ol-dehydrogenase. The transfer of the double bond from position 5 to 4 necessitates the participation of an isomerase. The dehydrogenase and isomerase are two different enzyme systems although both reactions take place simultaneously; the reversibility of each was demonstrated by incubating an acetone powder of sheep adrenal microsomes in the presence of androst-4-ene,3,17,dione as substrate and NADPH as cofactor.[42]

Microsomal 21-hydroxylase catalyzes the transformation of progesterone to deoxycorticosterone. This reaction takes place in the presence of molecular oxygen and NADPH.[43,44] The 21-hydroxylase is inhibited by carbon monoxide, and light at 450 nm can reverse this inhibition.

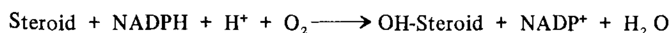
Deoxycorticosterone travels from microsomes to mitochondria where 11β-hydroxylase catalyzes its transformation to corticosterone. This reaction takes place in the presence of NADPH and molecular oxygen and can be inhibited by carbon monoxide.[45]

Aldosterone possesses an aldehyde group on angular carbon-18. It is not yet completely clear whether aldosterone is derived directly from corticosterone or indirectly with 18-hydroxycorticosterone as an intermediary. The mitochondrial oxygenation at position 18 of corticosterone is dependent on the presence of molecular oxygen and NADPH: this reaction can also be inhibited in the presence of carbon monoxide.[46,47] The transformation of 18-hydroxycorticosterone to aldosterone in vitro by adrenocortical tissue was shown possible although the yield is small compared to that from corticosterone under the same conditions.[48,49] Sandor et al.[47] reported no transformation of exogenous 18-hydroxycorticosterone to aldosterone by duck adrenal; results of their kinetic studies suggest that aldosterone and 18-hydroxycorticosterone originate simultaneously from corticosterone. It is interesting to note that, in all the species studied, adrenal aldosterone biosynthesis is always accompanied by that of 18-hydroxycorticosterone. In solution, 18-hydroxycorticosterone is in the cyclic 18,20 hemiketal form; this species is more resistant to oxidation than the open α-ketol and may be also more resistant to dehydrogenation. Consequently, we have to be careful in interpreting low aldosterone formation yield when 18-hydroxycorticosterone is used as substrate in vitro; it is quite possible that in vivo conditions might differ considerably and be much more propitious to the dehydrogenation of 18-hydroxycorticosterone.

Although corticosterone is believed to be the main precursor of aldosterone, it is not an obligatory intermediate in the conversion of progesterone to aldosterone. Progesterone and deoxycorticosterone were shown to be substrates that can be hydroxylated in position 18 and serve as precursors of aldosterone in normal and tumoral human adrenal cortex.[50] 18-Hydroxy-deoxycorticosterone was isolated and identified from rat adrenal cortex.[51] Frog adrenocortical slices were shown to be able to transform radioactive 18-hydroxyprogesterone and 18-hydroxy-deoxycorticosterone to aldosterone.[48] It was demonstrated that addition of radioinert 18-hydroxycorticosterone or corticosterone could inhibit the transformation of labeled progesterone to aldosterone, while 18-hydroxy-deoxycorticosterone showed no inhibition when added to incubation media of rat adrenal, suggesting that 18-hydroxy-deoxycorticosterone is not an important intermediate in aldosterone synthesis.[52] 18-Hydroxy-deoxycorticosterone is largely secreted by zona fasciculata-reticularis and, to a lesser extent, by zona glomerulosa. Adrenal blood flow is centripetal from the periphery, and 18-hydroxycorticosterone formed by inner zones — although not excluded — has little opportunity to serve as substrate for aldosterone formation.

C. Mechanism of Adrenal Steroid Hydroxylation

The transformation of cholesterol to aldosterone is effected by a series of enzymatic transformations which consists primarily of the mixed-function oxidase type as defined by Mason.[53] One atom of an oxygen molecule reacts to form a hydroxyl group in the steroid substrate while the other oxygen atom and one molecule of hydrogen generate water. The stochiometry of the basic hydroxylation reaction has been proposed as follows:[54]



$$\text{Steroid} + \text{NADPH} + H^+ + O_2 \longrightarrow \text{OH-Steroid} + \text{NADP}^+ + H_2O$$

Oxygen is a stable molecule due to the spherical symmetry of its electron distribution; therefore, it must be activated before incorporation into the steroid substrate. It is generally accepted that cytochrome P-450 is the oxygen activator involved in most, if not all, of the steroid hydroxylations in the adrenal cortex. This component was shown

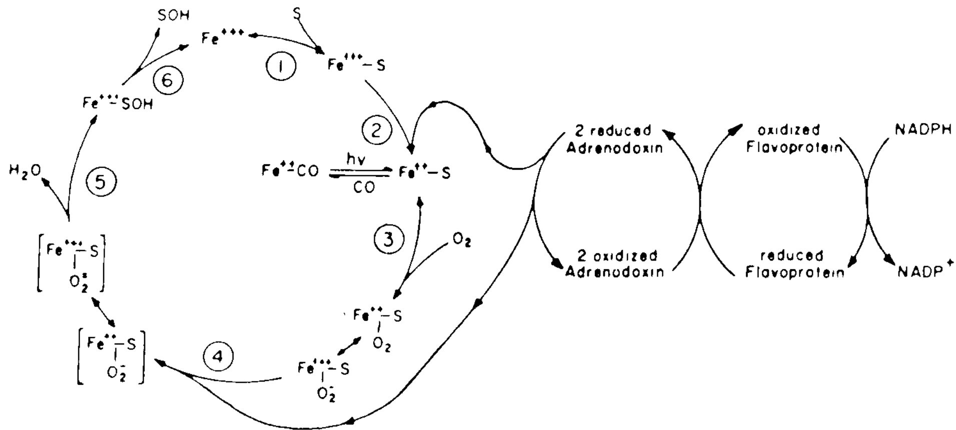


FIGURE 4. Schematic representation of the role of cytochrome P-450 and adrenodoxin in the electron transport reactions of adrenal cortex mitochondria associated with steroid hydroxylation. (From Estabrook, R. W., Mason, J. I., Baron, J., Lambeth, D., and Waterman, M., *Ann. N.Y. Acad. Sci.*, 212, 27, 1973. With permission)

to participate in the cholesterol side-chain cleavage reaction,[55] the 21-hydroxylation,[44,54,56,57,60] the 11β-hydroxylation,[58,59,61,62] and in the oxygenation of position 18.[46,47,63] This cytochrome combines with carbon monoxide to form a complex with absorption maximum at 450 nm, giving rise to the term "P-450."[64]

In mitochondria, cytochrome P-450 is the terminal component of an electron transport chain composed of three units bound to membranes:[59,61,65,67] (1) a flavoprotein: adrenodoxin-NADPH reductase, (2) a nonheme-iron protein: adrenodoxin, and (3) a hemoprotein: cytochrome P-450. The flavoprotein has a molecular weight of 54,000 daltons with one FAD molecule per mole protein. Adrenodoxin was purified to crystallization and contains 114 aminoacid residues, 2-g atoms of iron, and 2 mol labile sulfur per mole protein. Its molecular weight is 12,500 daltons.[65] Adrenodoxin is believed to be used twice to reduce cytochrome P-450 during mitochondrial hydroxylation reaction.[56,65] Figure 4 is a schematic of the hypothetical mechanism of adrenal mitochondrial steroid hydroxylation proposed by Estabrook et al.[56] The mitochondrial cytochrome P-450 responsible for the 11β-hydroxylation seems to differ from that involved in the cholesterol side-chain hydroxylation;[66,68] however, both enzyme systems are located in the inner membrane of mitochondria.[69] The mitochondrial 18-hydroxylase system is labile[47] and, consequently, there is no data available for a purified system. Microsomal cytochrome P-450 is different from that of mitochondria. On sucrose density-gradient centrifugation, microsomal cytochrome P-450 remains associated with the smooth surface fraction.[69] Hydroxylations of steroid by adrenal microsomal preparations necessitate the participation of an electron transport chain consisting of a flavoprotein but not adrenodoxin. As shown in Figure 5, a role for cytochrome b_5 and an unknown electron carrier has been postulated in the reduction of cytochrome P-450.[56,70]

The lack of inhibition of 21-hydroxylation in beef adrenal cortex microsomes by antiserum to mitochondrial adrenodoxin established that this iron sulfur protein has no significant role in the microsomal system.[71] However, the inhibition of the 21-hydroxylation of progesterone by antiserum to liver microsomal NADPH-cytochrome c reductase demonstrated the specificity and necessity for the microsomal reductase.[72]

In addition to its role as oxygen activator, cytochrome P-450 binds the steroid substrates prior to their hydroxylation; it is postulated that the element responsible for enzyme specificity in relation to the steroid position to be hydroxylated is provided by

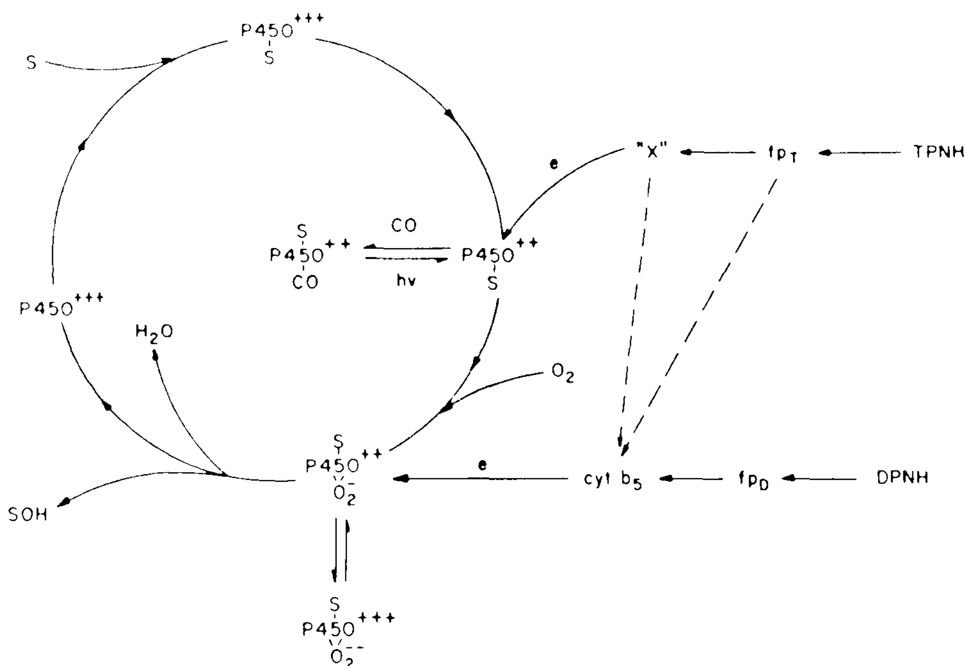


FIGURE 5. Schematic representation of electron transport reactions associated with the function of cytochrome P-450 in microsomes. (From Estabrook, R. W., Mason, J. I., Baron, J., Lambeth, D., and Waterman, M., *Ann. N.Y. Acad. Sci.*, 212, 27, 1973. With permission.)

cytochrome P-450. The hemoprotein appears to exist in low and high spin states and many factors can provoke interstate transitions. In general, hemoproteins with a Soret band near 420 nm are low spin hemoproteins while those with a Soret band near 390 nm are high spin; the spin state correlates well with optical spectra. The binding of cytochrome P-450 with steroid substrates produces typical absorbance patterns with a maximum at 385 nm and a minimum at 420 nm (Type I spectrum) or a minimum at 385 nm and a maximum at 420 nm (modified Type II spectrum).[73] The cytochrome P-450 spectrophotometric characteristics associated with its binding with carbon monoxide and with different steroid substrates offer a relatively easy approach to the study at the molecular level of the properties of this pigment.

V. ROLE OF IONS

A. Potassium

In many circumstances, potassium functions *in vivo* as a major regulator of aldosterone secretion. Small variations in the plasma potassium concentration within the physiological range were shown to provoke significant changes in aldosterone secretion. A small increase in plasma potassium concentration, irrespective of a diminution in sodium concentration, was able to stimulate considerably the secretion of aldosterone in man[74-76] and in experimental animals.[77,78-81] The rat adrenal zona glomerulosa width decreased under dietary potassium restriction and increased under potassium loading.[82] Morphological changes were not observed at the zona fasciculata-reticularis, indicating that only the zona glomerulosa was affected by potassium intake. Small changes in the potassium concentration in incubation media in vitro are sufficient to significantly influence the production of aldosterone in isolated adrenal cortical tissues.[28,83]

Potassium seems to have a stimulating effect at an early step of aldosterone biosynthesis. This ion was reported to stimulate reactions preceding the formation of pregnenolone[28,84,85] and that of corticosterone.[86] Capsular adrenal glands from potassium deficient rats converted 57% less deoxycorticosterone to 11β-hydroxylated corticosteroids than the analogous preparation from potassium-repleted animals.[86] The activities of the final steps of aldosterone synthesis are also affected by variations in potassium concentration. Dietary potassium deficiency provoked a 30-fold decrease in the conversion in vitro of corticosterone to aldosterone by rat adrenal compared with tissues of potassium-repleted animals.[87] This conversion returned to normal after 24 hr of normal potassium intake. Haning et al.[88] also observed an increased conversion of [^{3}H] corticosterone to [^{3}H] aldosterone by capsular gland cells stimulated by potassium.

In nephrectomized, decapitated dogs, marked increases in aldosterone secretion were observed in response to small changes in serum potassium concentration.[78,80,89] Under the condition of sustained elevation of arterial blood pressure and suppression of plasma renin activity by continuous infusion of angiotensin II, dog adrenal remained very sensitive to changes in serum potassium concentration. Plasma aldosterone increased when potassium chloride was infused together with angiotensin II. These studies suggest that potassium has an effect on the adrenal zona glomerulosa cells which is not mediated through ACTH or the renin-angiotensin system.

Isolated adrenal cells were recently used to study the effect of potassium on the formation of aldosterone. Short- and long-term experiments were performed. In isolated cells prepared from rat adrenal capsular preparation, the production of aldosterone and corticosterone increased with increasing potassium concentration in the incubation media.[90] No aldosterone production was detected when dog adrenal glomerulosa cells suspension were incubated in the absence of potassium.[91] However, a significant increase in production occurred when even a weak potassium chloride concentration (0.5 m*M*) was added to incubation media. Maximal steroidigenic response was obtained at 8.0 m*M* potassium chloride. Higher potassium concentrations did not increase the aldosterone formation. In intact adrenal cells[92] and zona glomerulosa cells of rat maintained in culture,[93] the aldosterone secretion was not maintained indefinitely by potassium. It was suggested that the loss of aldosterone secretion could have been due to the absence of an unknown "glomerulotrophic" agent.

The hypothesis that the effects of stimuli (i.e., ACTH, angiotensin II, sodium depletion, and hyperkalemia) known to promote aldosterone production are mediated by changes in intracellular potassium has created considerable interest.[94] Recent observations show that in the absence of potassium angiotensin II and ACTH had no effect on aldosterone formation in isolated canine glomerulosa cells.[95] Potassium appeared to be an obligatory requirement for the action of angiotensin II and ACTH. Indeed, a significant enhancement of both the sensitivity and response of the canine adrenal glomerulosa cells to ACTH and angiotensin II by potassium was demonstrated. The action of potassium was not on the angiotensin II receptor binding, since no effect on either binding affinity or binding capacity of the adrenal receptor sites could be observed.

Szalay[96] has presented evidence that angiotensin II in vitro altered the ionic gradient between the adrenal cells and the surrounding medium, with a decrease in tissue sodium and increased tissue potassium content. This author suggested that angiotensin II affects the active sodium-potassium transport mechanism. It is of interest that the greater amount of Na^+-K^+-activated ATPase is found in the zona glomerulosa zone of the adrenal cortex as compared with that of the zona fasciculata-reticularis.[97] In addition, aldosterone formation was reported to be inhibited by ouabain, which is an inhibitor of the Na^+-K^+-ATPase.[98] Intracellular potassium concentration was measured in rat adrenal zona glomerulosa dispersed cells purified by unit gravity sedimentation.[98]

The stimulation of these glomerulosa cells by angiotensin II and serotonin to produce corticosterone resulted in no significant changes in intracellular potassium concentration, thus showing no correlation between intracellular potassium ion content and increased steroidogenesis. When the potassium in the medium was decreased, corticosterone production was stimulated and intracellular potassium concentration was increased. This should not be taken to mean that intracellular potassium has no role in the control of aldosterone production but, rather, that it cannot be the sole major factor.

The role played by cyclic-AMP in the control of corticosteroid synthesis in the zona glomerulosa in relation to potassium is not well defined. It is known, however, that in isolated bovine adrenal glomerulosa cells the addition of dibutyryl cyclic-AMP stimulated the synthesis of aldosterone.[99] The induction of cyclic-AMP formation by small changes in potassium concentration were reported in ox adrenal cortical slices[83] and in rat adrenal capsular tissue.[97] Recently, potassium ion was shown to stimulate cyclic-AMP formation in dispersed capsular rat adrenal cells purified by fractionation on a bovine serum albumine gradient;[100] 3.6 to 8.4 m*M* potassium stimulated similarly both corticosterone and cyclic-AMP accumulation in incubation media. Above an 8.4 m*M* potassium concentration, corticosterone production decreased whereas the cyclic-AMP concentration was maintained. This suggests that potassium exerts a dual effect and causes a decrease in corticosterone that is independent of cyclic-AMP formation.

B. Sodium

Changes in plasma sodium concentration influence the secretion of aldosterone. The reports in this field draw unanimous conclusions: sodium depletion is a strong stimulus to the secretion of aldosterone[28,80,101-110] while sodium loading inhibits it. The mechanism of action of sodium on the adrenal cortex is complex due to the interrelationships existing between this ion and other factors involved in the regulation of aldosterone. For example, sodium could act directly per se on adrenals, or through the renin-angiotensin system, or through changes in plasma potassium or ACTH concentrations. It is also possible that sodium acts in conjunction with all these factors.

Sodium depletion in vivo produces an hypertrophy of the adrenal zona glomerulosa[28,82] and increases the capacity of this tissue to subsequently synthesize aldosterone in vitro.[111] Homogenates of adrenals of rats maintained a low sodium diet transformed exogenous cholesterol to corticosterone and aldosterone more actively than did control material from animals kept on a normal diet.[106] Isolated adrenal mitochondria of rats maintained on a low sodium diet were shown to metabolize more corticosterone to aldosterone than did mitochondria from control animals.[112]

At first, a direct influence exerted by sodium on the adrenal cortex was suggested; however, this effect was subsequently shown not to be very strong. Using adrenal autotransplants in sheep, Blair-West et al.[80] concluded that the sodium ion has only a small aldosterone-stimulating effect. Other researchers observed that sodium could affect aldosterone biosynthesis; a decrease of sodium concentration from 148 to 73 meq/ℓ resulted in a 40% increase of aldosterone production in rat adrenal quarters incubated in vitro.[28] Similar observations were reported for beef adrenal slice preparations; large changes of sodium concentration in the incubation media were necessary to produce a relatively small increase in aldosterone secretion.[83] It was also shown that high sodium concentrations lower the binding affinity of beef adrenal mitochondrial cytochrome P-450 for corticosterone.[113] This could possibly explain the diminution of aldosterone secretion in sodium loading experiments.

As slight potassium concentration changes were sufficient to induce an increase of aldosterone secretion and plasma potassium was increased when rats were fed on a low sodium diet, it was suggested that the in vivo increase of the aldosterone secretion

in these sodium-depleted animals was mediated through an increase in the potassium level.[81] In the rat, studies on the combined effect of potassium and sodium have shown that the increased aldosterone secretion due to sodium depletion was not inhibited by simultaneous potassium depletion. In addition, sodium-depleted and potassium-loaded animals synthetized significantly more aldosterone than animals which were only potassium loaded.[108] Similar results were obtained in sheep: a low sodium diet or high potassium intakes independently increased aldosterone secretion. The greatest stimulation occurred with both a low sodium and high potassium intake.[80] These data show that sodium depletion affects the aldosterone secretion through mechanisms other than the elevation of potassium level.

The renin-angiotensin system is an important mechanism through which sodium can stimulate the formation of aldosterone. There is a known relationship between sodium balance and renin activity; sodium restriction increases plasma renin activity[102] and elevates the level of plasma angiotensin II.[114] In addition, rat adrenal glands were shown to be more sensitive to angiotensin II under sodium depletion.[115] However, the role played by the renin-angiotensin system does not sufficiently explain increased aldosterone secretion during sodium deficiency, as increases in aldosterone synthesis can occur under low sodium-deficiency stimulation in nephrectomized animals.[116] Also, sodium depletion may increase aldosterone secretion in sheep without a significant change of blood angiotensin II level.[105] It was reported that short-term infusion (2 to 24 hr) of angiotensin II to conscious rats increased aldosterone secretion[117] while no increase was noted when the infusion lasted for more than a week.[118] Similarly, angiotensin II infusion to conscious dogs resulted in an increase of aldosterone secretion that lasted a few hours.[79] These studies therefore suggest that angiotensin II might well be one of the mechanisms through which aldosterone secretion is regulated with short-term sodium restriction but not necessarily on long-term sodium restriction.

The response of adrenal to stimulus by ACTH to synthesize aldosterone is increased by sodium restriction. In dogs kept on a low sodium diet, ACTH stimulated early stages but not final stages in the pathway of aldosterone synthesis, as was the case for angiotensin II.[119] Two sites on the aldosterone biosynthetic pathway are known to be stimulated by sodium restriction, one before[28,106,120-122] the formation of corticosterone and one after.[28,112,121-124] Changes in activities of enzymes involved in the transformation of cholesterol to pregnenolone and of corticosterone to aldosterone are, presumably, mainly responsible for the increased aldosterone formation when the organism is stimulated by sodium restriction.

VI. RENIN-ANGIOTENSIN SYSTEM

An aldosterone-stimulating substance (A.S.H.) secreted by the kidney juxta glomerular apparatus has been described.[125,126] This substance was subsequently shown to correspond to renin and to act on the adrenal zona glomerulosa morphology as well as on aldosterone secretion.[127,128] The mechanism of action of renin is not completely understood, but it seems that this substance acts by the intermediary of angiotensin II. When released, renin catalyzes the cleavage of angiotensinogen to angiotensin I. The renin substrate is a plasma α2-globulin synthesized by the liver. The decapeptide angiotensin I is converted to angiotensin II through the action of a converting enzyme in the plasma.[129]

It was shown that injection of the octapeptide angiotensin II resulted in an increase in aldosterone secretion in man,[130-133] dog,[79,134,135,137] and sheep.[80,134-136] The induced secretion of aldosterone in isolated dog and sheep adrenal by small doses of angiotensin II appears to be greater than the total concentration of aldosterone in normal glands.[134,135] This indicates that angiotensin II stimulates the production of aldosterone as well as its release.

In the rat, results have been inconsistent; first, no stimulation of aldosterone secretion by angiotensin II was reported by various researchers.[118,138,139] On the contrary, an increase of aldosterone production was observed following the perfusion of angiotensin II through rat adrenals.[140-142] Chronic treatment with angiotensin II for 1 to 4 weeks increased both the secretion, and adrenal content of aldosterone and also produced histological modifications in the adrenal, which resulted in a widening of the zona glomerulosa.[143] In the conscious rat, however, only small physiological quantities of infused angiotensin II were necessary to stimulate aldosterone secretion. Surgical preparations and anesthesia considerably diminished the secretion of aldosterone in response to infusion of angiotensin II.[117] In long-term experiments, chronic infusion of angiotensin II that maintained high blood pressure in intact conscious dogs increased the secretion of aldosterone, which returned to the control value after 6 hr;[79] similar results were obtained with sheep.[144] In conscious rats, as above mentioned, short-term infusion of angiotensin II stimulated the secretion of aldosterone.[117,142] Elevated values were not sustained on long-term experiments (1 to 2 weeks), but when potassium instead of angiotensin II was infused, sustained aldosterone production was observed.[79]

It was reported that the addition in vitro of angiotensin II during incubation of rat adrenals fed a normal diet failed to stimulate aldosterone production.[141,145] However, Müller[146] reported a slight stimulation of aldosterone synthesis in adrenal quarters from rats maintained on a low sodium diet when high angiotensin II concentration was added to incubation media. No stimulation could be observed when decapsulated adrenal glands were used.[28] The in vitro stimulation of aldosterone secretion from endogenous precursor by angiotensin II was demonstrated in beef adrenal preparations, both with high angiotensin II doses (100 μg/g of tissue) and low doses (0.1 μg/g of tissue).[147,148] High angiotensin II doses could also stimulate the formation of cortisol and corticosterone while low physiological concentrations could only stimulate aldosterone synthesis.[148] A direct in vitro effect of angiotensin II on aldosterone production was also reported in the dog.[134]

A direct action of angiotensin II was tested for in adrenal cell cultures. The inability of angiotensin II to enhance rat adrenal cells to synthesize aldosterone was first reported.[149] A stimulating action was subsequently found by other groups. With purified rat glomerulosa cells, Bing et al.[150] observed high aldosterone secretion responses to concentrations of angiotensin II that were only slightly higher than normal plasma levels. Using cultured adrenal cells from species other than the rat, it was also possible to demonstrate increases of aldosterone production when physiological doses of angiotensin II were added to bovine,[99,151] rabbit,[151] and dog[91,95] cells in culture. In bovine glomerulosa cells, angiotensin II stimulated the production of aldosterone in the absence of detectable changes in the cyclic-AMP level.[99] Aldosterone response of canine glomerulosa cell suspensions were 10- to 20-fold more sensitive to angiotensin II than to ACTH. The maximal response (three to eight times the basal level of aldosterone) was induced with 3×10^{-10} M angiotensin II, and a decrease was observed with higher concentrations. The presence of potassium ion was shown necessary for the action of angiotensin II, suggesting a modulating action of this ion upon adrenal sensitivity to the hormone.[95]

All available information indicates that angiotensin II acts at the level of the first steps of the conversion of cholesterol to aldosterone.[76,84,147,148] In the rat, angiotensin II stimulated the in vitro transformation of [^{3}H] cholesterol to [^{3}H] aldosterone, while there was no stimulation observed when [^{3}H] pregnenolone, [^{3}H] deoxycorticosterone, or [^{3}H] corticosterone were used as exogenous tracer substrates.[84] In bovine adrenal, an enhancement of labeled acetate incorporation into aldosterone was observed during stimulation with angiotensin II but not when [^{14}C] cholesterol was used.[152] Thus, al-

though there is a concensus concerning the site of action of angiotensin II at an early step in the synthesis of aldosterone, the precise point is not yet known.

An effect of angiotensin II at a late step of aldosterone biosynthesis has also been proposed.[87,119,124] It was suggested that this effect could be mediated by the increased corticosterone made available by the stimulation of angiotensin II at an early biosynthetic step.[87] In the dog, however, the conversion of corticosterone to aldosterone was reported to be significantly increased by the infusion of angiotensin II into sodium-depleted animals,[119] while ACTH failed to induce the same phenomenon. Infusion of different doses of angiotensin II in dogs maintained on a normal sodium diet failed to show the same effect in the late pathway. Another proof of the effect of angiotensin II on adrenal cortex is the evidence for the presence of specific receptors for angiotensin II in this tissue.[153-156]

Angiotensin III (angiotensin heptapeptide 2-8) can be formed by the action of an aminopeptidase on angiotensin II.[157] Angiotensin III was shown to have a greater relative activity than angiotensin II in stimulating the biosynthesis of aldosterone.[142,151,156,158,159] Adrenal glomerulosa cells have the enzymatic system necessary to convert angiotensin II to angiotensin III,[156] and angiotensin III could be detected in glomerulosa cells after their exposure to radioactive angiotensin II.[156] In addition, the heptapeptide was shown to bind to an adrenal receptor with greater affinity than angiotensin II, although there is no proof for a specific angiotensin III receptor. These data are extremely interesting and raise many questions concerning the possible role that could be played by angiotensin III in the regulation of aldosterone formation at the zona glomerulosa cell level.

It seems that the mechanism of aldosterone regulation by angiotensin II at the adrenal level differs from that of ACTH. However, as with ACTH, angiotensin II requires the presence of Ca^{++} to express its full effect.[83] Forman et al.[160] established that [1-sarcosine, 8-alanine] angiotensin II is a specific inhibitor of angiotensin II but has no effect on the stimulation of aldosterone synthesis by ACTH. These results suggest that ACTH and angiotensin II have separate receptor sites on the adrenal zona glomerulosa cells. It was also reported that angiotensin II could stimulate the steroidogenesis in bovine adrenal section[83] and in bovine adrenal glomerulosa cells[99] without increasing the production of cyclic-AMP. With purified rat glomerulosa cells, Tait et al.[90] could stimulate the formation of aldosterone by angiotensin II without noticing any increase in cyclic-AMP. However, the participation of cyclic-AMP in the mechanism of angiotensin II action cannot be ruled out by these experiments since "active" cyclic-AMP concentration could probably change inside adrenal without being detectable.

The control exerted by angiotensin II is a part of a system involving the juxta-glomerular cells of the kidney, liver, and adrenal cortex. All conditions favoring an increased renin secretion (e.g., the obliteration of the kidney arteries by the technique of Goldblatt[234]) provoke an increase in the angiotensin II production, resulting in an elevation in blood pressure and in aldosterone production.[161] In the regulation in this complex system, the notions of plasma volume and electrolytes concentration also play a role. In high concentration, sodium can eliminate the steroidogenic action of angiotensin II,[162] while sodium depletion can increase the angiotensin II production.[163] It seems that the role of angiotensin II in the maintenance of arterial tension is also determined, in part, by the plasma sodium level and the plasma volume.[161]

VII. ADRENOCORTICOTROPIC HORMONE

A. ACTH and Aldosterone Synthesis

ACTH is known to regulate adrenal glucocorticoid synthesis;[165] its short-term action on the formation of aldosterone in vivo and in vitro has also been reported in many species, including man.[28]

ACTH can stimulate corticosteroid production for a short period of time and maintain corticosteroidogenesis over a long time period. The immediate effect of ACTH is the enhancement of the specific synthesis of glucocorticoids within 15 min; the site of this short-term action is probably at the level of the transformation of cholesterol to pregnenolone, which is a limiting step in steroid synthesis.[165] Over a long period of time, ACTH would maintain the integrity of the adrenal cortex (mainly in the zona fasciculata) by controlling the growth and division of mitochondria[164] and the proliferation of the smooth endoplasmic reticulum.[165] This long-term effect does not maintain the aldosterone secretion that returns to basal levels after a few days of chronic ACTH treatment. In addition, hypophysectomy, which almost completely abolished the glucocorticoid secretion, provokes only a small reduction of the secretion of aldosterone. ACTH administration to hypophysectomized animals, however, returns aldosterone secretion to normal.[28]

Studies have demonstrated that the administration of ACTH to human subjects fed a low sodium diet leads to a significant increase of aldosterone secretion. This action was pronounced in sodium deficiency and negligible when the dietary sodium was in high concentration. It was recently observed, however, that a small quantity of ACTH (21 ng/min for 60 min) infused in normal human subjects on an unrestricted sodium intake resulted in a marked increase in plasma aldosterone level in each case. No changes were observed in either the plasma angiotensin II or plasma potassium concentrations during this increased aldosterone secretion, suggesting that ACTH acted directly on the zona glomerulosa.[166] Very interesting results were reported by Hata et al.:[167] after the suppression of endogenous ACTH production by the administration of dexamethasone, insulin-induced hypoglycemia failed to provoke a rise in plasma aldosterone and cortisol while plasma renin activity was increased; however, aldosterone increased in response to the stimulation by exogenous ACTH in those subjects pretreated with dexamethasone.

A circadian pattern of plasma aldosterone concentration exists in supine normal subjects;[168,169] a similar diurnal cycle was reported for plasma renin activity, and it has been suggested that fluctuations of plasma aldosterone concentrations are under the control of the renin-angiotensin system.[170] The plasma aldosterone diurnal rhythm also parallels changes in plasma cortisol levels, which leaves open the possibility that ACTH might be responsible for both cycles.[171] Another possibility is that a central mechanism could control the secretion of both renin and ACTH.

In isolated canine adrenal glomerulosa cells, 10^{-10} *M* ACTH significantly stimulated the formation of aldosterone with maximum stimulation obtained at 10^{-8} *M*. At physiologic concentration (10^{-11} *M*), ACTH did not stimulate the synthesis of aldosterone.[91] Production of aldosterone was shown to be highly dependent on the presence of potassium; no stimulation by ACTH could be obtained without its addition.[95] In monolayer culture of rat adrenal zona glomerulosa, ACTH first stimulated the formation of aldosterone; in long-term experiments, however, the aldosterone formation was decreased in the presence of ACTH.[149] The same effect was observed in vivo when high plasma ACTH levels were maintained over several days.[172]

The in vitro response of rat adrenal preparations to ACTH could be experimentally modified by changes in sodium and potassium intake. ACTH stimulated the formation of aldosterone in adrenal preparations from rats maintained on a low sodium diet; preparations from rats kept on a high sodium or on a low potassium intake produced less aldosterone than their respective controls when incubated in the presence of ACTH.[28]

The concept that ACTH acts first at the adrenal membrane cell level and subsequently affects the permeability of the mitochondria membrane has gained support in recent years. Lefkowitz et al.[173] have demonstrated a specific binding of ACTH in a

purified adrenal cell preparation; ACTH stimulated the adenyl cyclase system of this preparation. Through the action of cyclic-AMP, the mobilization, entry, and transformation of cholesterol to pregnenolone is facilitated. ACTH needs the presence of calcium for its action.[174-176] The overall mode of action of ACTH is not simple, and many events occur when adrenals are stimulated by this hormone. As demonstrated by Castells et al.,[177] ACTH provokes the formation of a rapidly labeled RNA during perfusion of an adrenal preparation. ACTH also acts at many other cellular levels, as was well demonstrated by the works of Kowal[178] and Garren et al.[179] on the mechanism of action of ACTH and that of Bartova et al.[180] on adrenal glycolysis. ACTH also stimulates blood flow through adrenocortical tissue; this increases both the quantity of substrates passing through the adrenal and the rate of removal of hormones secreted by the gland.

Colby et al.[181] demonstrated that, in the rat, hypophysectomy increased 5α-reductase activity of the adrenal cortex and diminished corticosterone secretion, while the injection of growth hormone provoked a decrease of 5α-reductase activity and an increase in corticosterone secretion. The activity of the cholesterol side-chain scission system is considerably diminished 24 hr post hypophysectomy. ACTH administration to these rats restored the activity of cholesterol side-chain cleavage to the initial value within 48 hr; corticosterone secretion increased but remained lower than normal. A longer ACTH treatment continued to increase the corticosterone secretion level without further affecting the activity of the cholesterol side-chain cleavage.[182] This implies that ACTH could act at other sites than that involved in the transformation of cholesterol to pregnenolone. It was suggested that the 21-hydroxylase activity could be stimulated in vitro by ACTH in adrenal gland segments of fetal rhesus monkey.[183] the basal level of 21-hydrxylation of 11β-hydroxyprogesterone was increased in rat adrenal preparations treated with ACTH for 4 days; however, this enhanced 21-hydroxylation could have been the consequence of an activation of the hexose-monophosphate shunt.[184]

Haning et al.[88] reported that adrenal (glomerulosa) cells converted more [^{3}H] corticosterone to [^{3}H] aldosterone when stimulated with ACTH than did control cells. In this case, the authors believe that the augmented conversion observed could have been due to a contamination by fasciculata cells. Moreover, Baumann and Muller[185] reported that, in the rat, the activity of the enzymes involved in the conversion of corticosterone to aldosterone appeared to be unaffected by the functional status of the pituitary.

B. Protein Synthesis

De novo protein synthesis appears to be an integral part of the mechanism of action of ACTH on adrenal steroidogenesis.[186,187] It is well known that ACTH stimulates the in vitro incorporation of amino acids into adrenal proteins. The short-term action of ACTH seems to be mediated through cyclic-AMP and the rapid synthesis of short-lived proteins of both mitochondrial and extra-mitochondrial origins.[188,189] The long-term action of ACTH on the components of steroid hormone systhesis in adrenal glands is less well known. We have recently performed long-term experiments (up to 8 hr) on the incorporation of radioactive leucine and aldosterone secretion in rat adrenal gland.[190] Male rats of the hooded strain of the same age were sacrificed, and adrenal glands were removed and cleaned in iced Krebs-Ringer bicarbonate medium containing no calcium. Glands were cut into quarters and distributed in tubes and then preincubated in the above-mentioned medium for 60 min at 37°C. The quarters were then transferred to a Krebs-Ringer bicarbonate medium containing 1.5 m*M* calcium and radioactive leucine. ACTH (0.5 unit per hour) was constantly added to the incubation media while buffer was added to the control series. The tubes were incubated for 1, 2, 4, or 8 hr. On termination of the incubation, the quarters were rinsed and

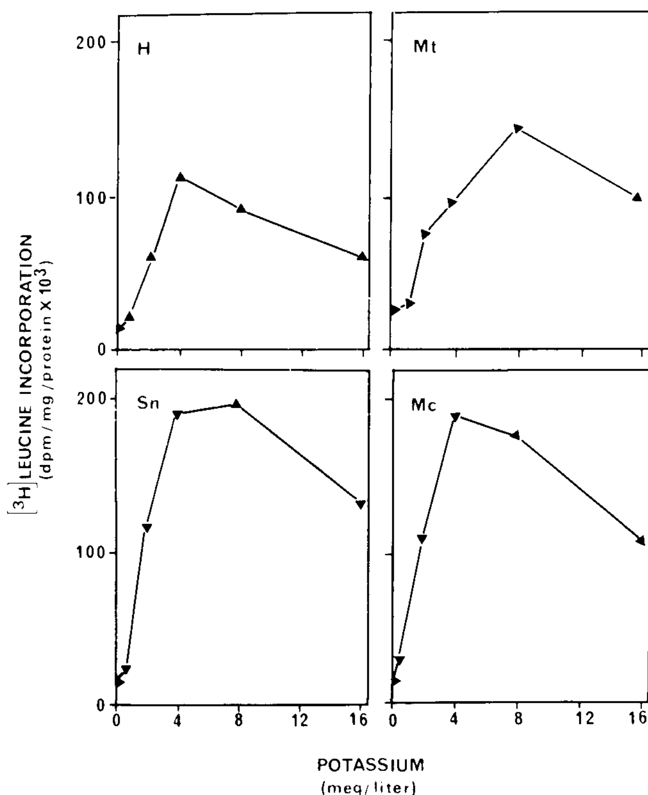


FIGURE 6. Effects of increasing amounts of potassium on [^{3}H] leucine incorporation into the protein of subcellular fractions of rat adrenal quarters incubated in the presence of ACTH (Lachapelle, M. and Lehoux, J.-G., *Ann. ACFAS,* 43, 75, 1976.)

homogenized in 0.05 phosphate buffer containing 0.25 *M* sucrose. Differential centrifugation was used to obtain subcellular fractions, which were precipitated with trichloroacetic acid. Protein concentration and radioactivity content of precipitates were then determined. The presence of ACTH provoked maximal stimulation of radioactivity incorporation into homogenate mitochondrial, microsomal, and supernatant fractions after 8 hr of incubation.

The effect of potassium on the ACTH stimulation of leucine incorporation was also studied.[191] Rat adrenal quarters were preincubated in a buffer that contained no potassium or calcium. Incubations were performed in media containing radioactive leucine, calcium, and from 0 to 16 meq of potassium per liter. ACTH was constantly infused to this series of tubes. The effect of increasing amounts of potassium in incubation media in the presence of ACTH is shown in Figure 6. Slopes of the curves are very abrupt at potassium concentrations from 0 to 4 meq/*l*. In the absence of potassium, ACTH did not produce any stimulation of leucine incorporation. Potassium was shown to be necessary during the whole incubation period to produce a full ACTH stimulation; withdrawal of potassium at any time during incubation resulted in lower incorporation values. Analyses of mitochondrial fractions performed by double dimension of electrofocusing and electrophoresis on polyacrylamide gel showed that certain protein bands were diminished in size when potassium was withdrawn from incubation media. These data show that, in long-term experiments, small physiological changes in the potassium concentration of media were sufficient to produce large variations in the incorporation of leucine into adrenal protein under ACTH stimulation.

Under similar experimental conditions, angiotensin II had no stimulatory effect on leucine incorporation. When added to the incubation media, prostaglandins E_2 and $F_{2\alpha}$ slightly increased leucine incorporation in the rat adrenal quarters. However, these two prostaglandins partly inhibited the stimulatory effect of ACTH when concomitantly added with ACTH to the incubation media. The aldosterone content of media at the end of incubation was increased by the presence of ACTH and potassium, compared to controls containing no ACTH. The increased aldosterone content was partly suppressed when prostaglandins E_2 and $F_{2\alpha}$ were added. This seems to indicate that ACTH, angiotensin II, and prostaglandins have different modes of action in the adrenal.

C. Cytochrome P-450

ACTH is an important factor in the maintenance of adrenal cytochrome P-450. In the rat, Purvis et al.[192] have demonstrated that the half-life of the adrenal cytochrome P-450 after hypophysectomy was about 4 days. The half-lives of the 21-hydroxylase, 11β-hydroxylase, and cholesterol side-chain cleavage system were equally shown to be of the same order of magnitude. ACTH administration to hypophysectomized rats partially restored the adrenal cytochrome P-450 content and the activities of 11β-hydroxylase and 21-hydroxylase. Jefcoate et al.[193] reported that ACTH facilitates the metabolism of cholesterol by increasing the proportion of the cholesterol-cytochrome P-450 complex; presumably, this could result from the transformation of a preexisting "inactive" form of cytochrome P-450 to the "active" state. Even for a short term (10 min), the administration of ACTH to hypophysectomized rats provoked increments of both the pregnenolone-cytochrome P-450 binding absorption spectra at 420 nm and the corticosterone secretion.[194,195] Mitochondria of rats stressed by ether anesthesia presented a cholesterol side-chain cleavage activity higher than that of unstressed controls. Spectra observed for the binding of pregnenolone to cytochrome P-450 were also higher for stressed rat adrenal preparation than control.[196]

In experiments on bovine adrenal mitochondria in vitro, it was observed that variations in the medium could produce substantial changes in the binding of corticosterone to cytochrome P-450. Conditions of pH and temperature producing optimal in vitro transformation of corticosterone favored the binding of corticosterone to this cytochrome.[197,198]

The condensation of glycine and succinyl-CoA to form δ-aminolevulinic acid is the first step of the heme biosynthesis. The presence of mitochondrial δ-aminolevulinic acid synthetase has been demonstrated in rat and bovine adrenocortical tissues.[199] Adrenocorticotropin and starvation were shown to increase adrenal δ-aminolevulinic acid synthetase activity in the rat.[199] We have observed[200] that administration of ACTH to rats 2 hr before sacrifice enhanced the capacity of adrenal homogenates to incorporate radioactive aminolevulinic acid in the mitochondrial heme fraction. This could indicate that ACTH has a regulatory effect on the adrenal heme synthesis and, presumably, on that of the hemoprotein cytochrome P-450.

VIII. PROSTAGLANDINS

It is known that prostaglandins influence adrenocortical steroidogenesis, but in vivo and in vitro experiments have given contradictory results concerning the role played by these substances. Peng et al.[201] reported that the administration of PGE_1 to rats resulted in adrenocortical ascorbic acid and cholesterol depletion and an increase of plasma corticosterone; a lack of effect for PGA_1 and $PGE_{2\alpha}$ was noted. As it was not possible to demonstrate stimulation by prostaglandins in hypophysectomized rats, they concluded that these substances acted to stimulate the secretion of ACTH and, conse-

quently, were not directly involved at the adrenocortical tissue level. Other experiments, however, showed that certain types of prostaglandin could directly affect the formation of glucocorticoids and that of aldosterone in the adrenal. Flask et al.[202] reported an increase in corticosterone production following superfusion of adrenocortical tissue from hypophysectomized rats with small doses of PGE_1, PGE_2 and $PGE_{2\alpha}$.

In man, PGA_1 was reported to increase aldosterone secretion while no change in ACTH, renin or serum electrolytes was observed.[203] Contradictory results were also reported, as unaltered aldosterone excretion rate was found by Carr[204] after treatment with PGA_1. Zusman et al.[205] reported a fourfold fall in plasma PGA_1 after surgical removal of a renal cortical tumor from a human subject. This patient had suffered from hypertension for 20 years and subsequently became normotensive.

There is evidence that prostaglandins are almost totally removed from the circulation by one passage through the lung.[235] However, PGA_1 and PGA_2 are more resistant to the lung metabolism and, consequently, could very well be secreted by an organ to reach another organ, such as the adrenal, to exert their action on aldosterone synthesis.

Zusman et al.[206] found an inverse relationship between plasma prostaglandins and sodium concentrations: prostaglandin A plasma level falls in rats on a high sodium diet and increases in plasma in animals fed a low sodium diet. In superfusion experiments,[207] pharmacological levels of PGA_2 produced a significant stimulation of corticosterone synthesis but not of aldosterone by the capsular rat adrenal and no response by the decapsulated gland, indicating that glomerulosa cells are more sensitive to PGA_2 than those of the inner zones. However, both aldosterone and corticosterone production rates of adrenal glands from sodium-restricted rats were increased by PGA_2; a role for PGA_2 in the control of aldosterone secretion during sodium restriction was subsequently postulated. Blair-West et al.[208] reported that the infusion in vivo of PGE_1 to sodium-deficient sheep resulted in a rise in cortisol and corticosterone secretion rates; a fall in aldosterone secretion was noted although large variations were observed within animals tested.

Spät et al.[209] indicated that synthetic PGE_2 slightly but significantly stimulated aldosterone and corticosterone production in vitro by rat adrenocortical tissue. By superfusion of rat adrenal gland, it was also found that PGE_2 stimulated production, providing additional evidence in favor of a direct effect of this prostaglandin on glomerulosa cells.[207] Satura and Kaplan[210] showed that prostaglandins E_1 and E_2 significantly stimulated the synthesis in vitro of aldosterone and, to a lesser degree, that of cortisol in the outer slices of bovine adrenal tissue; PGA_1, $PGF_{1\alpha}$, and $PGE_{2\alpha}$ were ineffective under the same experimental conditions.

It is also possible that different types of prostaglandin could play antagonistic roles at the adrenal level. Indeed, Honn and Chavin[211] reported that human adrenals are stimulated in vitro by PGE_1 and PGE_2 to increase cyclic-AMP and cortisol synthesis at the same time as $PGF_{1\alpha}$ and $PGF_{2\alpha}$ inhibited the formation of cyclic-AMP and cortisol. Another proof of the action of prostaglandins in the adrenal is the presence of specific receptors in human and ovine cell membranes as shown by Dazord et al.[212]

As for ACTH, the presence of calcium was necessary for the action of PGE_1 on bovine adrenal,[210] and an increase in the adrenal cyclic-AMP level was noted; both events were inhibited by puromycin but not by actinomycin D. It is not clear if prostaglandins act independently or in conjunction with ACTH at the adrenal level. It is known, however, that the biosynthesis of prostaglandins from arachidonic acid by cat adrenocortical cells was enhanced by ACTH.[213] In cat adrenal cells,[214] steroidogenic concentrations of ACTH stimulated the release of PGE and PGF while indomethacine suppressed this ACTH-facilitated release; these findings support a link between prostaglandins and corticosteroidogenesis stimulation by ACTH.

The intracellular localization of prostaglandin synthetase in adrenal cells is not

known. Radioautography studies on rat adrenal cortices treated with tritiated PGE_1 revealed some radioactivity in the plasma membrane, in lipid droplets, and in other cellular organelles.[215] Cinti and Feinstein[216] reported the presence of cytochrome P-450 in human platelets. They found that the arachidonate stimulation of platelet-aggregating factor formation by isolated microsomes was inhibited by metyrapone and carbon monoxide, two cytochrome P-450 inhibitors. The binding of prostaglandins $F_{1\alpha}$, E_1, E_2, and arachidonic acid to bovine adrenal microsomal cytochrome P-450 was also reported.[217] Prostaglandins and arachidonic acid induced type II and type I spectral change, respectively. The binding affinities of prostaglandins, measured by their apparent dissociation constant, were of the same order of magnitude as those of natural steroids. These results suggest that a role could be played by cytochrome P-450 in arachidonate and prostaglandin metabolism.

In summary, there are good indications that prostaglandins are important factors in the control of adrenal cortex steroidogenesis. The exact mechanism of action of these substances is not known yet either at the fasciculata-reticularis or at the glomerulosa levels, and more work will be necessary before it will be possible to determine their real physiological importance.

IX. CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) may also play a role in the regulation of aldosterone secretion. In the rhesus monkey, a considerable increase in plasma aldosterone concentration was obtained by the stimulation of certain hypothalamic areas. This increase of aldosterone secretion was accompanied by an increase in plasma renin activity.[218] In rats kept on a normal diet, it was shown that the posterior lobe of the hypophysis contains factors stimulating the mitotic activity of the glomerulosa cells.[219,220] Payet and Isler[221] recently reported that vasopressin and, to a less extent, oxytocin were mitogenic while α-MSH and β-MSH were without effect. In sodium-deficient rats, it was shown[222] that lesions in the supraopticoneurohypophysial tract provoked a significant decrease in aldosterone production in vitro 5 days after the operation. Injection of arg-8-vasopressin 2 units per 4 hr for 2 days completely restored the in vitro aldosterone production. These data suggest that the CNS could also have a role to play in the control of aldosterone synthesis, the importance of which should not be disregarded.

X. PINEAL GLAND

It was initially postulated by Farrell and McIsaac[223] that the pineal area possessed a high adrenoglomerulotrophic activity capable of stimulating the secretion of aldosterone. However, this postulate was weakened as it could not be substantiated by Wurtman et al.[224] It has been also shown that the pineal gland secretes a substance having an inhibitory effect on the adrenal. In the absence of light, rat pineal gland appeared to secrete a substance that induced a decrease in the degree of compensary hypertrophy provoked by unilateral adrenalectomy; pineal extract had the same effect. This inhibitory effect was reversed by pinealectomy and ACTH injection.[225] These data suggest that the pineal gland could be a mediator between ACTH and environmental lighting. It was also reported that pinealectomy resulted in a stimulation of aldosterone production in the normal rat; however, plasma aldosterone concentration returned to normal 3 months after the operation.[226]

Among substances isolated from the pineal gland, melatonin was shown to have an inhibitory action on the adrenal cortex that could reverse the adrenal enlargement following unilateral adrenalectomy.[227] The pineal melatonin content follows a circardian rhythm, reaching a maximum 1 hr after dark on a controlled light-dark cycle.[227]

It was shown that melatonin acts directly on the rat adrenal cortex in vitro, presumably, to inhibit the enzyme activity that catalyzes the conversion of cholesterol to pregnenolone; melatonin also diminishes the response of the adrenal cortex to ACTH in vitro.[228] A hexane extract of bovine pineal gland was also shown to inhibit the transformation in vitro by the adrenal of [^{14}C]-labeled progesterone to 11β-hydroxylated metabolites;[229] this indicates that melatonin could act at more than one site in the biosynthetic steroid pathway in the adrenal.

5-Hydroxytryptamine (serotonin) is another substance found in the pineal. It preferentially stimulates the steroidogenesis of the zona glomerulosa. Rosenkrantz[230] was the first to report a specific stimulating action in vitro of serotonin on aldosterone synthesis. Many subsequent reports have shown that serotonin is, in fact, one of the most potent of in vitro aldosterone-stimulating substances.[28,231] The site of stimulation by serotonin on the aldosterone biosynthetic pathway is mainly located at the level of the conversion of cholesterol to pregnenolone; other sites of stimulation also exist between the formation of pregnenolone and that of aldosterone.[28] However, proof of an effect of serotonin on aldosterone synthesis in vivo is lacking; on the contrary, serotonin was reported to have little or no effect on the secretion of cortisol, cortisone, and aldosterone.[232] However, when administered in vivo, the concentration of serotonin reaching the adrenal might be negligible due to the large uptake of this substance by blood platelets.[233] This could possibly explain the nonstimulating effect of serotonin on aldosterone secretion when administered in vivo.

XI. CONCLUSION

The regulation of aldosterone synthesis is complex. As indicated in preceding pages, there are many control factors of aldosterone synthesis that act directly or indirectly on the adrenal gland, and interactions between the various involved factors do not facilitate the understanding of the action of a given control factor.

Potassium ion has surely a very important role to play in the control of the synthesis of aldosterone but it is not the only main factor. The renin-angiotensin system also seems to play an extremely important role; a decrease of the body sodium concentration stimulates this system to enhance the formation of aldosterone synthesis at the adrenal zona glomerulosa level. Following this event, the aldosterone formed acts at the kidney level to promote the excretion of potassium and the retention of sodium. From this, it can be seen that a very close interrelationship exists between the sodium ion that influences the synthesis of aldosterone and the control by aldosterone of the excretion of potassium and the retention of sodium.

Recent studies have indicated that ACTH plays a more important role in the control of aldosterone synthesis than was at first believed. There are also other factors such as prostaglandins, substances from the pineal gland and of neurohypophysial origin, that seem to contribute in the control of aldosterone synthesis. Sites of action of control factors on the aldosterone biosynthetic pathway are not completely known, and the mechanism of action of these substances at the molecular level is not understood. The study of the mode of action of control factors of aldosterone synthesis is challenging and should contribute very interesting findings.

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Chapter 3

FORMATION AND METABOLISM OF STEROID CONJUGATES: EFFECT OF CONJUGATION ON EXCRETION AND TISSUE DISTRIBUTION

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I. INTRODUCTION

The steroid hormones, like many other substances of endogenous and exogenous origin, are involved in a number of conjugation reactions in the body. It has been generally assumed that the conjugation of steroid hormones with glucosiduronic or sulfuric acid is a prerequisite for and a commitment to their biological inactivation and excretion. Clearly, this conjugation yields substances more water soluble than the free steroids, but the necessity of increasing solubility for excretion is questionable. Slaunwhite et al.[1] demonstrated that the plasma concentrations of estrogens, progesterone, and their metabolites do not exceed their aqueous solubility under normal conditions.

For many years, glucosiduronates, sulfates and sulfoglucosiduronates were thought to be the only steroid conjugates occurring naturally in mammals. More recently, however, a number of different conjugation reactions have been shown to occur. Methylation, acetylation, and phosphorylation of steroid hormones have been reported. Steroid hormones have also been shown to undergo conjugation with glutathione, N-acetylglucosamine, and glucose in vivo. The large variety of steroid conjugates, existing in both water-soluble (glucosiduronates, sulfates, and phosphates) and lipid-soluble (methyl and acetyl derivatives) forms, supports the suggestion that the conjugation process is not primarily a method for excretion, as had been proposed by early workers.

Another interesting feature, particularly of the estrogen conjugates, is that a conjugate may undergo hydrolysis and subsequent direct conversion to another conjugate without entry of the free steroid into the general pool. In addition, metabolism of the steroid nucleus may occur without hydrolysis. A number of different tissues participate in both the conjugation and the subsequent hydrolysis. This suggests that many conjugates may serve as an important source of biologically active steroids.

This chapter will deal primarily with the conjugation and subsequent metabolism of the steroid conjugates and the effect of these steps on transport, excretion, and tissue distribution. The major emphasis will be placed on the estrogen conjugates, because most of the available information relates to these conjugates.

II. TISSUES ACTIVE IN FORMATION OF STEROID CONJUGATES

Much of our knowledge about the formation of steroid conjugates by specific tissues has been gained using in vitro incubation and *in situ* perfusion techniques in a number of species, including man. Liver, kidney, small intestine, lung, breast, and the feto-placental unit have been shown to be active in the formation of steroid conjugates. Muscle, some endocrine organs such as the adrenal, ovary and testis, and the endometrium have also been reported to form steroid conjugates under in vitro conditions.

A. Liver

The liver is the most active organ in the conjugation of steroids and was assumed by early workers to be the sole site of steroid conjugation. In addition to its ability to conjugate a number of different steroids, the liver is also able to form several different types of conjugates. The liver of almost every species studied has the ability to form both steroid glucosiduronates and sulfates. Several species can form N-acetylglucosaminides, glucosides,[2] and glutathione conjugates,[3-9] as well as double conjugates.

Crepy first showed that estrone could be converted to estrone glucosiduronate by guinea pig liver slices in vitro.[10] Goebelsmann et al.[11] demonstrated that guinea pig liver forms only estriol-3-glucosiduronate from estriol in vitro. On the other hand, the human liver was shown to be highly specific for conjugation in the 16α-position, producing estriol-16α-glucosiduronate and 16α-hydroxyestrone-16α-glucosiduronate.[12-15]

The human liver also forms glucosiduronates of 17β-estradiol, 17α-estradiol, estrone, and a number of other steroids.[16] In fact, with the exception of estriol-3-glucosiduronate, the human liver has been reported to be capable of forming glucosiduronates of all steroids thus far studied, including pregnanediol,[17-19] testosterone,[20] and androsterone.[19] Liver preparations from the rat,[21-24] mouse,[23,25,26] dog,[23,27] guinea pig,[11,23,28] pig,[29] rabbit,[23,28,30,31] and some nonhuman primates[32-37] have been shown to be capable of forming steroid glucosiduronates.

The ability of the human liver to form 17β-estradiol-17-glucosiduronate is controversial. The formation of 17β-estradiol-17-glucosiduronate by the human liver in vitro was reported by Hobkirk et al.[38] On the other hand, Sa'at and Slaunwhite[39] reported formation of limited amounts of estrone and 17β-estradiol-3-glucosiduronate after incubation of human liver homogenates in the presence of excess uridine diphosphoglucuronic acid. However, a recent report questions the formation of 17β-estradiol-3-glucosiduronate by human liver homogenates.[38,40] Following in vitro incubation of human liver slices with radioactive 17β-estradiol, Hobkirk et al.[38] could not identify any C-3-monoglucosiduronates except after incubation with excess uridine diphosphoglucuronic acid.[38]

Nonhuman primates such as the baboon, chimpanzee, and rhesus monkey differ from the human in that the major metabolic product after incubation of estrone and 17β-estradiol with liver homogenates is 17β-estradiol-17-glucosiduronate.[33,34] Musey et al.[33] reported significant conversion of 17β-estradiol and estrone to 17β-estradiol-17-glucosiduronate after incubation with rhesus monkey liver homogenates in vitro. The three major conjugates formed after incubation with estrone were 17β-estradiol-3-glucosiduronate, estrone glucosiduronate, and 17β-estradiol-17-glucosiduronate in the ratio 2:1:4.5. All primate livers studied conjugate estriol in a similar manner. For example, baboon,[32,35] rhesus,[33] chimpanzee,[36] and human[39] all form estriol-16α-glucosiduronate exclusively.

The steroid uridine diphosphoglucuronyl transferases are associated in most tissues with the microsomes.[34,41,42] These transferases may be solubilized with heat-treated snake venom.[43] Some reports indicate that the steroid uridine diphosphoglucuronyl transferases are ubiquitous in the subcellular fractions of mammalian tissues.[16,34,44-46] Enzymes capable of forming glucosiduronates of estrogens have been found in the ground plasma and cytosol fractions from the liver and gastrointestinal tract of the human.[44,46] A similar distribution was noted in various tissues from the pig.[16,46] Glucuronyl transferases which synthesize 17β-estradiol-17-glucosiduronate as well as small amounts of estrone and 17β-estradiol-3-glucosiduronates were found in the microsomal, 150,000-g residue and cytosol fractions.[34]

The sulfokinases of liver are particularly active in the conjugation of phenolic and neutral steroids in all species studied under appropriate in vitro conditions. DeMeio et al.[47-49] reported sulfokinase enzymes capable of conjugating estrogens in rat liver. These same workers incubated dehydroisoandrosterone with the cytosol fraction from rat liver and obtained dehydroisoandrosterone sulfate.[50] While liver preparations from most animals have been demonstrated to form estrogen monosulfates, the rat and hen are unique in their ability to form disulfates of 17β-estradiol.[51-53] In the hen, the disulfate of 17β-estradiol was the major conjugate formed in vivo.[53]

Sulfate formation cannot be solely an inactivation and excretion process. The metabolic clearance rate is significantly lower for estrone sulfate than for free estrone.[54-56] This fact supports the hypothesis that the sulfates may function as a method for storage of steroids in the blood.[57,58] Furthermore, the presence of sulfatases capable of hydrolyzing estrone sulfate in a number of tissues suggests that blood estrone sulfate may serve as a source of physiologically active estrogens.[59]

Other workers have suggested that the estrogen sulfates are intermediates in the metabolism of estrogens.[60] Fishman et al.[61] reported evidence that estrone sulfate is the

primary metabolite of 17β-estradiol and may be the principal intermediate to other metabolites. They found that when radioactive 17β-estradiol was administered simultaneously via the oral and intravenous routes, part of the oral dose was immediately conjugated with glucosiduronic acid; this conjugate was not transformed to other metabolites oxygenated at C-2 and C-16.[61] A greater part of the oral dose mixed with the intravenous hormone and was transformed to all the metabolites of estradiol with a constant isotope ratio. This portion was concluded to be estrone sulfate because estrone sulfate and all other sulfate metabolites showed a constant isotope ratio.

Another group of steroid conjugates formed primarily in the liver is the double conjugates, of which the sulfoglucosiduronates are the principal example in the human. Estriol-3-sulfate 16α-glucosiduronate constitutes a significant portion of the total conjugated estriol present in plasma and urine at term in human gestation.[62-65]

Unlike other species, the rabbit excretes injected estrone and 17β-estradiol almost exclusively as the double conjugate, 17α-estradiol-3-glucosiduronate, 17-N-acetylglucosamine.[30,66] In a careful in vitro study of the formation of this double conjugate, it was found that the N-acetylglucosamine could be transferred to the 17α-hydroxyl groups only after prior conjugation of the 3-phenolic group with glucosiduronic acid.[30,66] Rabbit liver microsomes have also been shown to transfer N-acetylglucosamine to the 3-sulfate form of 17α-estradiol and 16α-hydroxyestrone but not to the free estrogens.[67] In addition, rabbit liver microsomes have been reported to form diglucosiduronates of 17β-estradiol, estriol, and 17-epiestriol.[68]

The transfer of glucose from UDP-glucose to phenolic and alcoholic groups of estrogens can be accomplished by liver microsomal preparations of various species.[36,69-71] Some interesting comparative differences exist both in the specificity of the glucosyl transfer to various hydroxyl groups and in the requirement for previous conjugation of the estrogen with glucosiduronic acid prior to addition of glucose to a second hydroxyl group. Rabbit liver and kidney microsomes were found to possess two separate steroid glycosylating enzymes.[69] One effects the transfer of glucose or galactose to the phenolic 3-hydroxyl group of estrone, 17α-estradiol, and 17β-estradiol, but not to estriol. The other is highly specific for the transfer of glucose to the 17α-hydroxyl group of 17α-estradiol, but requires the prior conjugation of the phenolic 3-hydroxyl group with glucosiduronic acid.[69] No transfer of glucose or galactose to steroids without an aromatic A-ring has been detected in the rabbit.

Microsomes of sheep liver can effect not only the glucosylation of the 17α-hydroxyl group of 17α-estradiol-3-glucosiduronate, but also 17α-estradiol and some other steroids containing a 17α-hydroxyl group, including epitestosterone.[70] No transfer of galactose to steroids has been detected with sheep liver.

In contrast to the sheep, microsomes of the human liver that readily form the 17-glucoside or galactoside of 17α-estradiol, but do not glucosylate the 17α-group of 17α-estradiol-3-glucosiduronate.[71] Chimpanzee liver microsomal enzymes can also transfer glucose and galactose to the 17α- but not the 17β-hydroxyl group of estradiol.[36] The chimpanzee resembles the sheep and the rabbit in its ability to form a 17-glucoside, but not a galactoside, of 17α-estradiol-3-glucosiduronate. The chimpanzee liver microsomes also have a relatively high capacity to form the 3-glucoside of estrone but no capacity to form the 3-glucosides of either 17α- or 17β-estradiol.[36]

The rabbit is the only other species which has thus far been found to form a 3-glucoside of estrone; it can also glucosylate the 3-hydroxyl of 17α- and 17β-estradiol.[69] Furthermore, the ability to form glucosides in the rabbit is relatively low in comparison to glucosiduronate formation under similar conditions, whereas in the chimpanzee the glucoside/glucosiduronate ratio is high.[36] In this regard, Jirku and Layne[72] reported the presence of significant amounts of a conjugate in chimpanzee urine after injection of ^{14}C-estrone, which was hydrolyzed by glucosidase (almond emulsion) but not by β-

glucuronidase and had solvent partition characteristics similar to a nonacidic monoglycoside.

The mammalian species show great differences in their ability to transfer glucose to estrogens. In the case of the chimpanzee, evidence has been presented which suggests that the ability of liver tissue to transfer glucose to a steroid hydroxyl group exceeds its ability to transfer glucosiduronic acid.[36] This indicates that the formation of glucosyl conjugates may be the preferred pathway over the formation of glucuronyl conjugates in some instances. Therefore, it appears unlikely that glucosyl transfer to alcohols and phenols by mammals is simply an alternative mechanism that acts only in the absence of uridine diphosphoglucosiduronic acid.[73]

In vitro preparations of rat liver have been demonstrated to methylate catechol estrogens at both the 2 and 3 positions.[74] The catechol-O-methyl-transferase of human liver[75] and placenta[76] also methylates free catechol estrogens at both phenolic positions in vitro.

Estrone and 17β-estradiol are converted by rat liver preparations to water soluble products which are neither sulfates nor glucosiduronates but peptide conjugates (glutathione) of 2-hydroxyestrone and 2-hydroxyestradiol-17β.[76,77] When 17β-estradiol was incubated with rat liver homogenates, C-1 and C-4 thio-ether conjugates of 2-hydroxyestradiol-17β and 2-hydroxyestrone were isolated.[76,77] Identification of the thiol linkage showed that the steroid and cysteine moiety of glutathione were connected through this linkage.

Using a liver perfusion technique, Quamme et al.[78,79] demonstrated that the chicken liver has enzymes capable of forming both sulfates and glucosiduronates of estrone and 17β-estradiol. These results confirmed previous in vitro and in vivo results which demonstrate that the major metabolites are the monosulfates.[53,80,81] Small amounts of the monoglucosiduronates as well as the diconjugates, disulfates diglucosiduronates, and sulfoglucosiduronates are also present.

B. Kidney

Reports from several laboratories suggest that renal tissues are capable of forming steroid conjugates. Diczfalusy et al.[81-83] identified estrogen conjugates in the human fetus after perfusion or intra-amniotic administration of radioactive 17β-estradiol and estriol. Estrone sulfate and 17β-estradiol sulfate were found in the lung, liver, and kidney. However, it was not clear from these studies whether these sulfates were actual synthetic products of the individual tissues. However, Wengle observed that both the isolated fetal lung and kidney could sulfurylate estrogens and deoxycorticosterone.[84] On the other hand, other workers were unable to demonstrate sulfurylation of steroids in the rat kidney in vitro.[85-87] The rat kidney was also demonstrated to form steroid glucosiduronates in some early studies.[88,89] The dog kidney has likewise been shown capable of glucosiduronate formation after in vivo perfusion with androsterone and etiocholanolone.[90]

The role of the primate kidney in the formation of estrogen glucosiduronates has been investigated by several workers. Kirdani et al.[91] demonstrated formation of estriol-16α-glucosiduronate by both human and baboon kidney following in vivo and in vitro perfusions and in vitro incubations with radioactive estriol. These workers were unable to demonstrate in vitro conjugation of estrone. However, Hobkirk et al.[92] later reported small but significant formation of estrone and 17β-estradiol glucosiduronates by human kidney homogenates. These preparations also converted 52 to 91% of radioactive estriol exclusively to estriol-16α-glucosiduronate. Rhesus monkey kidney has also been shown to form estrogen glucosiduronates in vitro.[93]

The rabbit kidney, like the liver, is unusual in that it possesses significant steroid N-acetylglucosaminyl transferase and steroid glucosyl transferase activity as well as steroid glucuronyl transferase activity.[66] Although the activity is lower in the kidney

than the liver, these enzymes were shown to have similar specificity and biochemical characteristics. In a like manner, human kidney tissue was shown to possess steroid N-acetylglucosaminyl transferase and glucosyl transferase activity, as well as steroid glucuronyl transferase activity.[94,95]

Following infusion of the kidney, 20% of free aldosterone was removed by the kidney, but only 1 to 3% of this appeared in the urine.[96] In contrast, greater amounts of the acid labile conjugate were found in the urine than could be expected from the renal extraction of this metabolite, leading the authors to conclude that the excess conjugates represented formation and direct excretion by the kidney.[96]

C. Intestine

Until Hartilia and co-workers[97-100] demonstrated that the intestinal mucosa was an important site of conjugation in the rat, the assumption had prevailed that conjugation of estrogens took place primarily in the liver. Radioactive estrone, 17β-estradiol, or estriol were incubated with duodenal mucosa of the rat and shown to form the respective estrogen glucosiduronates.

This observation was extended to the human intestinal tract by Diczfalusy and coworkers[101-103] who showed that various parts of the gastrointestinal tract convert free estrogens into both phenolic and alcoholic glucosiduronates. Extensive work by Breuer and his colleagues[44,45,104-106] led to the identification in the microsomal fraction of the human intestine of glucuronyl transferases capable of forming estrone glucosiduronate, the 3- and 17β-monoglucosiduronates of 17β-estradiol, the 3-, 16α-, and 17β-monoglucosiduronates of estriol, and the 3- and 16α-monoglucosiduronates of 16α-hydroxy-estrone. A glucuronyl transferase capable of forming estriol-16α-glucosiduronate was also found in the ground plasma and cytosol fractions.

D. Lung

Few data are available on the contribution of the lung to the formation of steroid conjugates. Human fetal and neonatal lungs have been reported to convert estrogens,[81,82] corticosterone, cortisone, deoxycorticosterone, and progestins[107,108] to their corresponding sulfates. However, because the fetus is generally rich in sulfotransferases which diminish progressively after birth, it is doubtful that the sulfotransferase activity is present in the adult.

Adult bovine lung homogenate has been reported to convert minute quantities of estrone to estrone sulfate.[109] However, following perfusion of a dog heart-lung preparation with radioactive cortisol, no evidence for conjugate formation was found.[110] In fact, there is evidence that the adult dog lung is capable of hydrolyzing estrogen conjugates to free estrogens.[59] Collins et al.[59] determined the arterio-venous differences of free estrogens and estrogen conjugates across the lung during constant infusion of radioactive estrone or estrone glucosiduronate. They found net uptake of estrone glucosiduronate and net production of free estrone and 17β-estradiol by the lungs and concluded that the lung was, in fact, a site for estrone glucosiduronate hydrolysis. (See section on steroid hydrolysis.)

E. Adrenal

Baulieu et al.[64,111-113] first isolated dehydroisoandrosterone sulfate from an adrenal tumor and showed that it was also secreted by normal and hyperplastic adrenal tissue. In a series of in vivo studies involving sampling of adrenal vein blood, direct evidence of dehydroisoandrosterone sulfate secretion from normal and pathological adrenals was obtained by comparing the concentration of the steroid sulfate in adrenal venous and peripheral vein blood taken simultaneously.[111]

The formation of dehydroisoandrosterone sulfate and other steroid sulfates by the adrenal has been demonstrated using various in vitro adrenal tissue

preparations.[107,108,114-121] Killinger and Solomon[114] incubated normal human adrenal homogenate with 7α-pregnenolone-^{3}H and isolated dehydroisoandrosterone sulfate, 17α-hydroxypregnenolone sulfate, pregnenolone sulfate, and 5-pregn-enetriol sulfate from the incubation extract. When 7α-pregnenolone sulfate was incubated under identical conditions, no evidence for metabolism of this conjugate was obtained. This suggested that 5-pregnenetriol and its sulfate may be secretion products of the normal human adrenal gland.[114]

III. ENDOGENOUS LEVELS OF STEROID CONJUGATES

Measurement of endogenous steroid conjugates in body fluids other than urine has received little attention because of the inadequate sensitivity of the available chemical methodology and the destruction of the original structure when concentrations of free steroid are determined after hydrolysis of the conjugate. The separation of conjugates into bulk "sulfate" and "glucosiduronate" fractions is easily achieved using thin-layer chromatography,[122] solvent partition,[123] Sephadex LH-20 chromatography,[124] Sephadex G25 chromatography,[64] or sequential enzyme hydrolysis.[125] These procedures, when monitored for methodological losses, permit accurate measurement of steroids conjugated at only one position, such as dehydroisoandrosterone, estrone, and pregnenolone.

However, these sulfate-glucosiduronate "fraction" separations are totally inadequate for the quantitation of individual conjugates of steroids with two or more hydroxyl groups. Sophisticated chromatographic systems have recently been devised to purify individual steroid conjugates on Sephadex LH-20,[126] alumina,[127] DEAE-Sephadex,[128,129] sequential Sephadex systems,[130] and by gas chromatography of the acetylated conjugate.[131] These methods have generally been applied only to estrogen conjugates, although the techniques should prove useful for other steroid conjugates.

Several groups have developed specific radioimmunoassays for the measurement of intact steroid conjugates. This approach appears advantageous in that, not only is a specific conjugate measured, but laborious chromatographic procedures and subsequent variable hydrolyses are avoided. In addition, the sensitivity of the radioimmunoassay permits accurate quantitation in small samples of biological fluid.

A. Pregnanes

Jänne et al.[124] identified several neutral steroid sulfates in plasma by gas-liquid chromatography and mass spectroscopy after purification of mono- and disulfate fractions on Sephadex LH-20. The following steroid conjugates were identified and quantitated: pregnenolone sulfate (30 to 70 ng/mℓ, female; 30 to 300 ng/mℓ, male); pregn-5-ene-3β,20α-diol sulfate (80 to 350 ng/mℓ, female; 80 to 440 ng/mℓ, male); pregn-5-ene-3β,20α-diol disulfate (60 to 150 ng/mℓ, female; 40 to 270 ng/mℓ, male); pregn-5-ene-3β,17α,20α-triol sulfate (30 to 80 ng/mℓ, female; $<$10 to 150 ng/mℓ, male). Similar findings were reported by Laatikainen et al.[132] using chromatography, solvolysis, and gas-liquid chromatography: pregnenolone sulfate, 60 to 110 ng/mℓ; pregn-5-ene-3β,20α-diol sulfate, 190 to 310 ng/mℓ; pregn-5-ene-3β,20α-diol disulfate, 60 to 200 ng/mℓ; and pregn-5-ene-3β,17α,20α-triol sulfate, 30 to 80 ng/mℓ. Of these C_{21} sulfates, only pregnenolone sulfate was shown to be secreted by the testis (testicular vein concentration 140 to 390 ng/mℓ).

The urinary and fecal excretions of progesterone metabolites were studied by Martin et al.[133,134] using sequential hydrolysis and gas-liquid chromatography. Their investigations showed that the administration of ampicillin to pregnant women caused a transient decrease in the urinary C_{21} glucosiduronates with no detectable effect on the sulfates or unconjugated steroids. The alteration in fecal excretion by ampicillin was of longer duration. Before treatment, free steroid constituted 69 to 79% of the progestin

in the feces with 19 to 20% present as glucosiduronates. After ampicillin administration, however, the sulfated form represented 28 to 44% of the total. These data emphasize the significant role of the enterohepatic circulation in steroid conjugate metabolism and demonstrate that alterations in bacterial flora of the intestine can have significant metabolic consequences.

Scommegna et al.[135] measured pregnenolone sulfate in maternal and fetal plasma at term. Their findings, summarized in Table 1, suggest that the fetus is the major source of pregnenolone sulfate. The 16α-hydroxylated metabolites of C_{21} steroid sulfates have also been studied in late pregnancy plasma.[136] In 11 women studied, the following monosulfates were found: 3α,16α-dihydroxy-5α-pregnan-20-one (8 to 80 ng/mℓ); 3β,16α-dihydroxy-5α-pregnan-20-one (6 to 55 ng/mℓ); 5α-pregnane-3α,16α,20α-triol (9 to 31 ng/mℓ); and 5α-pregnane-3β,16α,20α-triol (11 to 77 ng/mℓ). The concentration of these steroids as disulfates proved to be 5 to 50 times lower than the monosulfates.

Peltonen and Laatikainen[137] identified several C_{21} glucosiduronates by gas-liquid chromatography after enzyme hydrolysis of a "glucosiduronate" fraction from Sephadex LH-20. Mean plasma values ± SE were 3α-hydroxy-5β-pregnan-20-one (430 ± 80 ng/mℓ); 5β-pregnane-3α,20α-diol (4810 ± 340 ng/mℓ); 3α,16α-dihydroxy-5α-pregnan-20-one and 3α, 16α-dihydroxy-5β-pregnan-20-one (2230 ± 350 ng/mℓ); 3α,6α-dihydroxy-5β-pregnan-20-one (1660 ± 280 ng/mℓ); 5α-pregnane-3β,16α,20α-triol (870 ± 270 ng/mℓ); and 5β-pregnane-3α,16α,20α-triol (1450 ± 240 ng/mℓ).

Using radioimmunoassay following solvolysis, Aso et al.[138] measured the plasma levels of pregnenolone sulfate in 15 women throughout the menstrual cycle. In the midfollicular phase, the mean concentration was 95 ng/mℓ (range 75 to 120 ng/mℓ); at midcycle 78 ng/mℓ (range 54 to 112 ng/mℓ); and in the luteal phase, 138 ng/mℓ (range 110 to 174 ng/mℓ). There was a significant elevation in the plasma concentration of pregnenolone sulfate in the luteal phase when compared with midcycle levels. The concentration of the sulfate was generally 50 times that of the free steroid in plasma.

Determinations of glucocorticoid sulfate in maternal and fetal blood at term, presented in Table 2, suggest that the fetus is the primary source of this conjugate.[139] The urinary excretion of glucocorticoid conjugates is shown in Table 3. It is interesting that only cortisol sulfate, cortisone sulfate, corticosterone sulfate, and 11-dehydrocorticosterone sulfate are elevated in pregnancy urine.[140] Luttrell and Steinbeck[141,142] measured the urinary excretion of the glucosiduronates of cortisol and cortisone in normal men and women and in pregnancy. They found the excretion of cortisol glucosiduronate ranged from 16 to 100 μg/24 hr (n = 14) with a mean ± SE of 58.6 ± 6.2 in normal men and women. Cortisone glucosiduronate ranged from 55 to 120 μg/24 hr with a mean ± SE of 81.0 ± 4.8 μg/24 hr (n = 15). Both of these conjugates were elevated in Cushings syndrome and following ACTH infusion. No progressive elevation appeared in three women studied throughout pregnancy.[142]

B. Androgens

A specific antiserum has been developed against testosterone glucosiduronate-bovine serum albumin and used to measure the level of testosterone glucosiduronate in urine of men and women[143] (Table 4). The levels of testosterone glucosiduronate were variable in men, ranging from 95 to 545 μg/day.[143] The levels in normal women were also variable, ranging from 1 to 50 μg/day.[143] When testosterone glucosiduronate was measured throughout the menstrual cycle in eight normal women, a midcycle peak occurred in all subjects which coincided with the LH peak ± 1 day.[143] Six subjects showed a luteal phase peak and five showed a follicular phase peak. Testosterone glucosiduronate excretion was significantly elevated in hirsute women when compared with normal women.[144] In another study, Jones et al.[145] compared the excretion of testosterone

TABLE 1

Mean Plasma Levels of Pregnenolone Sulfate in Human Pregnancy at Term[135]

Source	n	ng/m*l* ± SE
Maternal plasma		
35—37 weeks gestation	11	135.3 ± 14.4
Labor	26	186.2 ± 16.2
Delivery	14	279.4 ± 34.9
Fetal artery	14	1700.0 ± 264.8
Fetal vein	14	1238.9 ± 177.2
Placenta intervillous space	8	371.2 ± 52.6

TABLE 2

Mean Concentration (ng/m*l* ± SD) of Pregn-4-ene C_{21}-yl Sulfates in Maternal and Fetal Plasma at Term[139]

	n	Mean ± SD (ng/m*l*)	Range (ng/m*l*)
Corticosterone sulfate			
Cord plasma	7	26 ± 11	19—52
Maternal plasma	7	1 ± 1	1—2
11-Deoxycorticosterone sulfate			
Cord plasma	7	34 ± 11	23—56
Maternal plasma	7	5 ± 1	3—7
11-Dehydrocorticosterone sulfate			
Cord plasma	7	17 ± 6	8—26
Maternal plasma	7	7 ± 2	3—11
Cortisol sulfate			
Cord plasma	4	5 ± 2	2—10
Maternal plasma	4	2 ± 1	1—2

TABLE 3

Mean Urinary Excretion of Corticosteroid C_{21} Sulfates During Pregnancy[140]

	Mean Urinary Excretion (μg/day ± SD) at:		
Steroid	Midterm	3rd trimester	Nonpregnancy
Tetrahydrocortisol glucosiduronate	798 ± 146	447 ± 96	989 ± 79
Tetrahydrocortisone glucosiduronate	1715 ± 341	1612 ± 392	2083 ± 234
Cortisol sulfate	53 ± 15	273 ± 70	33 ± 8
Cortisone sulfate	17 ± 5	46 ± 7	8 ± 1
Tetrahydrocorticosterone glucosiduronate	—	196 ± 58	120 ± 18
Corticosterone sulfate	86 ± 23	225 ± 28	39 ± 5
11-Dehydrocorticosterone sulfate	36 ± 8	83 ± 11	25 ± 2

TABLE 4

Mean Urinary Excretion (μg/24 hr ± SD) of Testosterone Glucosiduronate as Determined by Radioimmunassay Procedures

Subject	n	Mean ± SD (μg/24 hr)	Range (μg/24 hr)	Ref.
Men	20	273 ± 164	95—545	143
Follicular women	80	16 ± 8	3—31	
Midcycle women	80	20 ± 11	1—50	
Luteal women	64	23 ± 10	5—45	
Men	26	164 ± 51	97—346	144
Women	16	24 ± 10		
Hirsute women	26	57 ± 25	30—130	

glucosiduronate in premenopausal and postmenopausal women with breast tumors and showed no significant difference in excretion between women with benign lesions and women with cancer in either menstrual category.

Krawczynska et al.[146] measured the testosterone released in the urine of infants after sequential β-glucuronidase treatment (for glucosiduronates) and acid hydrolysis (for sulfates). Not only was the excretion quite high in these subjects, but the excretion of the sulfate conjugate was three to four times that of the glucosiduronate (mean ± SD of 6.15 ± 2.42 vs. 1.43 ± 0.62 μg/24 hr in the first 2 weeks of life and 22.68 ± 9.91 vs. 7.16 ± 2.5 μg/24 hr after 3 weeks). These levels of testosterone sulfate are higher than those reported for normal men (6 to 10 μg/24 hr).[147]

Testosterone released by β-glucuronidase was first identified by Burger et al.[148] in normal human plasma. Subsequently, Tresquerres et al.[149] developed a specific radioimmunoassay procedure for the measurement of testosterone glucosiduronate also in normal human plasma. Their data, presented in Table 5, indicate that the testosterone/testosterone glucosiduronate ratio in normal men is approximately 3.[149] However, in normal and hirsute women and hypogonadal males, the ratio is close to unity. This may be a reflection of the direct conversion of androstenedione and dehydroisoandrosterone to testosterone glucosiduronate.[149]

In a male subject undergoing catheterization for hypertension, the concentration of testosterone glucosiduronate in the testicular vein was not significantly elevated over that in the cubital vein,[149] suggesting that the glucosiduronate is not secreted by the testis but is a metabolic product. Interestingly, the infusion of 1000 IU hCG in six normal males produced no elevation of peripheral testosterone glucosiduronate after 180 min, whereas testosterone was significantly elevated after 90 min.[149]

The information available on testosterone sulfate has been reviewed.[150] Urinary excretion in normal men ranged from 2.6 to 23 μg/24 hr and in normal women from 1 to 5 μg/24 hr. Saez et al.[151] used solvolysis with quantitation by the double isotope derivative technique to measure testosterone sulfate in the plasma. In nine normal men, the plasma concentration of testosterone sulfate ranged from 0.66 to 2.36 ng/mℓ (mean ± SD = 1.44 ± 0.66 ng/mℓ) and in four women ranged from 0.13 to 0.38 ng/mℓ (mean ± SD = 0.24 ± 0.10 ng/mℓ). In a series of paired peripheral and spermatic venous plasma samples, Saez et al.[151] clearly demonstrated that testosterone sulfate is secreted by the testis. These workers found the concentration in the spermatic vein to be 5 to 30 times that in the peripheral vein, an observation later confirmed by Laatikainen et al.[132]

The findings with respect to the sulfate and glucosiduronate of testosterone may be summarized as follows. The free steroid is the principal plasma component in men; the plasma concentration of the glucosiduronate is greater than that of the sulfate.

TABLE 5

Mean Concentrations (ng/ml ± SD) of Testosterone and Testosterone Glucosiduronate in Human Plasma[149]

Subject	n	Testosterone	Testosterone-17β-glucosiduronate
Males	20	6.32 ± 2.27	2.10 ± 0.45
Females	17	0.43 ± 0.07	0.47 ± 0.18
Hirsute females	14	1.28 ± 0.55	1.36 ± 0.68
Hypogonadal males	9	1.82 ± 0.68	1.08 ± 0.80

The sulfate, but not the glucosiduronate, is secreted by the testis. In women, the plasma concentration of the free steroid is similar to that of the glucosiduronate, and both are greater than that of the sulfate. The urinary excretion of the glucosiduronate of testosterone is much greater than that of the sulfate in both men and women.

Three methods have been used for the determination of dehydroisoandrosterone-3β-sulfate in normal plasma: direct radioimmunoassay,[152] radioimmunoassay of dehydroisoandrosterone following solvolysis,[138,153,155] and gas-liquid chromatography following solvolysis.[124] In general, the results obtained from these techniques are similar; Table 6 presents data from several laboratories. A novel method employing solvolysis, purification, reduction to androstenediol, and quantitation by competitive protein binding to sex steroid binding globulin was reported by Andre and James.[157] Wang et al.[156] suggest that dehydroisoandrosterone-3β-sulfate is higher in men than in women, but this does not appear to be supported by the available data. The mean levels of dehydroisoandrosterone-3β-sulfate do not vary with respect to the phase of the menstrual cycle.[138] This steroid conjugate is apparently secreted by the adrenal but not to any great extent by the testis or ovary,[132] a finding which could account for the lack of variation with respect to sex or reproductive status. There does appear to be variation with age, the highest levels being reached at 20 to 30 years.[156] Hopper and Yen[158] have measured dehydroisoandrosterone and its 3β-sulfate during puberty and reported both a sex difference and a change in the ratio of the free to the conjugated form.

To determine if the alteration in androgen metabolism in women with breast cancer was due to alteration in the metabolism of dehydroisoandrosterone-3β-sulfate or androsterone-3α-sulfate, Wang et al.[156,160] studied the levels of these steroid sulfates in a series of breast cancer patients. They were unable to demonstrate a significant difference between preoperative patients and controls in any age category. The postoperative levels after breast surgery were significantly decreased in most cases. However, in 92 patients with advanced breast cancer, both dehydroisoandrosterone-3β-sulfate and androsterone-3α-sulfate were significantly decreased in all but one age category.

When normal women were treated with Sulpiride, a drug which increases plasma prolactin, a significant increase in plasma levels of dehydroisoandrosterone-3β-sulfate was demonstrated.[159] This finding is worthy of note, but the alteration in androgen level has thus far only been shown to be correlated with an increase in plasma prolactin. Many workers have tried unsuccessfully to demonstrate a direct effect of prolactin on androgen metabolism.

Nowaczynski et al.[161] studied the urinary excretion of the 3β-sulfate and 3β-glucosiduronate conjugates of dehydroisoandrosterone. The sulfate is the principal urinary conjugate, with a mean excretion ± SD of 2957 ± 304 μg/24 hr as compared with 617 ± 206 μg/24 hr of the 3β-glucosiduronate (n = 7). The excretion of the 3β-sulfate was severely depressed in six patients with essential hypertension and normal plasma renin activity, as well as in eight essential hypertensives with decreased plasma renin activity.

TABLE 6

Plasma Levels of Dehydroisoandrosterone Sulfate in the Human

Source	n	Mean ± SD (μg/mℓ)	Range (μg/mℓ)	Method	Ref.
Males	10	2.63 ± 0.63	1.94—4.05	Solvolysis	153
Females	6	2.64 ± 1.04	1.01—3.77	+ RIA	
Males	42	2.59 ±0.99	0.93—4.52	Solvolysis	155
Females	22	2.13 ± 0.77	0.81—3.58	+ RIA	
Males	16	2.41	0.68—3.47	Solvolysis	124
Females	7	1.63	0.80—2.40	+ GLC	
Females, day LH − 8	15	2.00	1.50—2.67	Solvolysis	138
Females, day LH − 0	15	2.12	1.55—2.89	+ RIA	
Females, day LH + 8	15	2.15	1.55—2.96		
Males	7	2.67 ± 0.56	1.99—3.34		
Females					
Premenopausal	8	1.62 ± 0.95	0.82—3.38		
Term pregnancy	8	0.62 ± 0.29	0.23—1.17	Direct RIA	152
Postmenopausal	6	0.30 ± 0.19	0.11—0.61		
Newborns — both sexes	8	2.69 ± 0.87	1.67—3.64		

The excretion of the 3β-glucosiduronate was greatly increased in the latter group. The significance of these findings is unclear at the present time.

The 16α-hydroxylated form of dehydroisoandrosterone-3β-sulfate plays a central role in steroid production by the feto-placental unit. The concentration of this steroid conjugate in maternal plasma and in the umbilical artery and vein has been measured by Simmer et al.[162] and Furuya et al.[163] The mean values ± SD were as follows: maternal vein, 267 ± 35 ng/mℓ[162] and 870 ± 220 ng/mℓ[163]; umbilical artery, 8141 ± 1112 ng/ /mℓ[162] and 4490 ± 2140 ng/mℓ;[163] and umbilical vein, 8663 ± 606 ng/mℓ[162] and 2970 ± 1450 ng/mℓ.[163] The disparity in levels reported by the two groups may result from differences in patient population or in methodology. However, the data confirm radioactive studies which show that the conjugate is produced in the fetus, released into the fetal circulation where it circulates largely unchanged, and is removed on the maternal side of the placenta, probably by conversion to estriol or a conjugate of estriol.

The 16β-hydroxylated form of dehydroisoandrosterone appears in the urine of men and nonpregnant women principally as the glucosiduronate (mean ± SD = 29.6 ± 5.9 μg/24 hr) but also as the sulfate (14.9 ± 4.0 μg/24 hr).[161] The excretion of both these conjugates was significantly elevated in patients with essential hypertension and normal plasma renin activity (glucosiduronate = 173 ± 43 μg/24 hr, sulfate = 110 ± 14.9 μg/24 hr) and in patients with essential hypertension and decreased plasma renin activity (glucosiduronate = 798 ± 129 μg/24 hr, sulfate = 600 ± 147 μg/24 hr).[161] The methodology employed, sequential hydrolysis, did not permit precise conjugate identification, since conjugation may be at the 3 or 16 position.

Androsterone-3α-sulfate has been measured in peripheral plasma following solvolysis by gas-liquid chromatography and by radioimmunoassay. Wang et al.[156] reported mean values of 547 ng/mℓ (range 50 to 1400) in 41 males and 559 ng/mℓ (range 70 to 1740) in 51 females. The mean values ± SD reported by Kream et al.[164] were higher: 743 ± 378 ng/mℓ (range 135 to 1540) in 27 males and 702 ± 419 ng/mℓ (range 216 to 1860) in 22 females. The ranges given are large but coincide, indicating that the differ-

ences reported by the two groups are probably caused by variations in populations. Neither group found sex differences in these plasma levels.

Other androgen sulfates have been identified in human peripheral plasma by gas-liquid chromatography after chromatography and solvolysis. Laatikainen et al.[132] found androstenediol monosulfate (40 to 90 ng/mℓ free steroid) and disulfate (80 to 160 ng/mℓ free steroid). Similar values for the monosulfate (30 to 190 ng/mℓ free steroid) and disulfate (70 to 580 ng/mℓ free steroid) were found by Jänne et al.[124] In addition, they measured epiandrosterone-3β-sulfate (30 to 430 ng/mℓ free steroid) and 5-androstene-3β, 17α-diol disulfate (30 to 150 ng/mℓ free steroid).[124]

C. Estrogens

The estrogen conjugate which has attracted the most attention has been and continues to be estriol-16α-glucosiduronate. Since this conjugate was demonstrated by Smith and Kellie[64] to be the major urinary estriol metabolite in late pregnancy, attention has centered on its potential for monitoring pregnancies. The quantitative importance of estriol-16α-glucosiduronate in urine was confimed by Ahmed and Kellie[130,165] and by Tikkanen.[166] Ahmed and Kellie[130] identified the following estrogens at 36 to 40 weeks gestational age (range of excretion rate; percent of total urinary estrogen): 16α-hydroxy-estrone-3-sulfate, 16-glucosiduronate (0.35 to 1.26 mg/24 hr; 0.9 to 2.8%); estriol-3-sulfate, 16-α-glucosiduronate (1.63 to 3.53 mg/24 hr; 5.3 to 8.0%); estrone glucosiduronate (0.13 to 1.77 mg/24 hr; 2.3 to 5.2%); 16α-hydroxyestrone-3-glucosiduronate (0.77 to 3.21 mg/24 hr; 2.5 to 6.1%); estriol-3-glucosiduronate (2.20 to 7.20 mg/24 hr; 6.8 to 16.2%); 17β-estradiol-17-glucosiduronate (0.27 and 0.50 mg/24 hr in two patients, not detected in others); 16-epiestriol-16-glucosiduronate (0.14 to 0.6 mg/24 hr, <1%); and estriol-16α-glucosiduronate (12.2 to 19.0 ng/24 hr, 36.7 to 43.2%). Tikkanen[166] found similar values for the urinary excretion of estriol conjugates at term: estriol-3-glucosiduronate, 2.6 to 14.8 mg/24 hr; estriol-3-sulfate, 16α-glucosiduronate, 0.45 to 4.68 mg/24 hr; estriol-3-sulfate, 0.3 to 1.77 mg/24 hr; and estriol-16α-glucosiduronate, 14.8 to 34.8 mg/24 hr. When the proportion of each estriol conjugate in each urine sample was calculated, a fairly constant ratio of 9:3:0.9:0.3 was found for estriol-16α-glucosiduronate/estriol-3-glucosiduronate/estriol-3-sulfate, 16α-glucosiduronate/estriol-3-sulfate at term.

In addition, four pregnancies were studied by Tikkanen[166] from 10 weeks to term. The urinary excretion of estriol-3-sulfate and estriol-3-sulfate,16α-glucosiduronate was less than 10% of the total estriol throughout the course of pregnancy. There was no consistent change in this proportion with increasing gestation. Estriol-3-glucosiduronate decreased from 30 to 40% of the total estriol at 10 weeks to 20% or less at term. In one patient studied, the proportion of estriol-16α-glucosiduronate excreted increased from 40% at 10 weeks to 80% at term. The other subjects showed a less dramatic increase in excretion of estriol-16α-glucosiduronate from 60% at 10 weeks to 70% at term.

Several laboratories have developed specific radioimmunoassays for the measurement of estriol-16α-glucosiduronate in pregnancy urine.[167-174] The values reported by different laboratories are similar, increasing from less than 1 mg/24 hr in early pregnancy to 40 to 50 mg/24 hr at term. The urinary excretion of estriol-16α-glucosiduronate from 57 different pregnant subjects is compared with the gestational age in Figure 1.[174] After about 30 weeks, the urinary concentration of estriol-16α-glucosiduronate rose progressively until term, reflecting the dependence of the fetal production of estriol on growth.

The excretion of estriol-16α-glucosiduronate has also been studied throughout the menstrual cycle by Lehtinen and Adlercreutz[171] and Wright et al.[175,176] The values reported are generally somewhat lower than those obtained by other investigators using acid hydrolysis and measuring total urinary estriol,[177,178] indicating that estriol-16α-

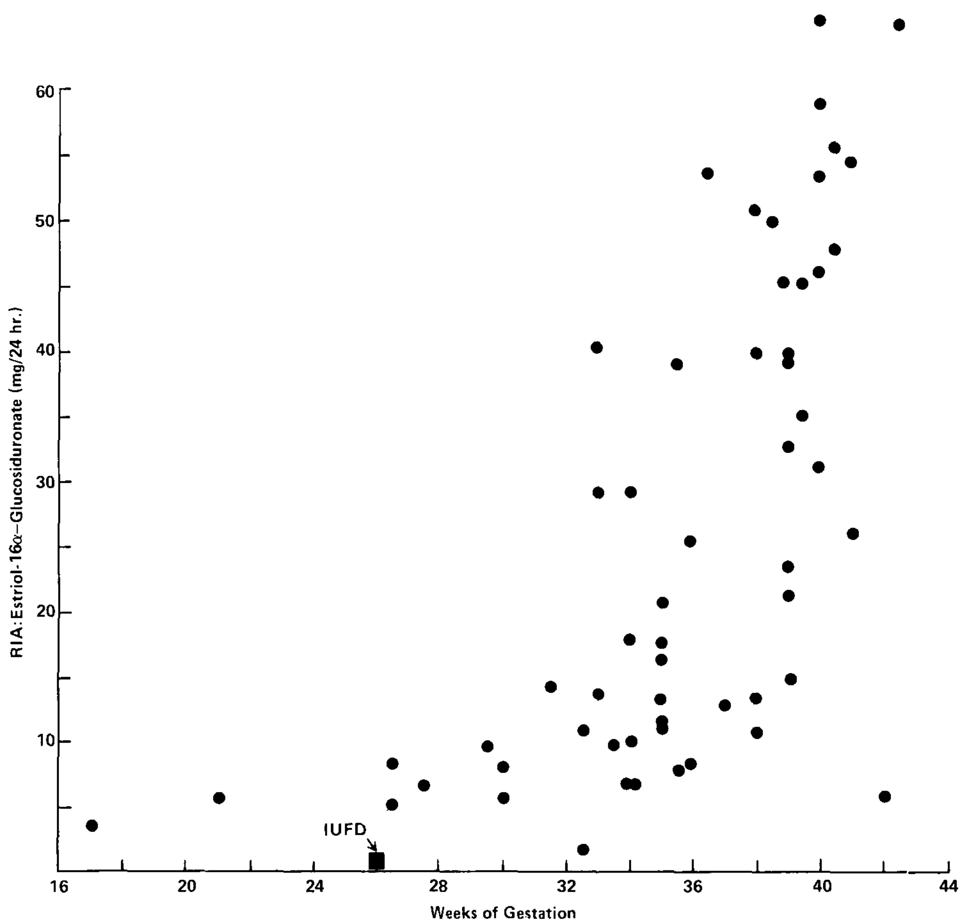

FIGURE 1. Urinary excretion of estriol-16α-glucosiduronate (mg/24 hr) from 57 pregnant women from the 17th week of gestation to term.[174]

glucosiduronate is not the only form of urinary estriol (and may not even be the major form) in the nonpregnant human.

The radioimmunoassay of estriol-16α-glucosiduronate has been applied to pregnancy plasma.[171,174,179] Lehtinen and Adlercreutz[171] followed two normal pregnancies throughout gestation; plasma estriol-16α-glucosiduronate gradually increased to 62 ng/mℓ and 118 ng/mℓ at term. Kerr et al.[179] studied five normal pregnancies, one with urinary tract infection and one with preeclampsia. When the estriol-16α-glucosiduronate levels were assayed in third trimester pregnancy plasma (Figure 2), the plasma levels exhibited a marked rise from 31 to 41 weeks of gestation.[174] Other workers reported a similar rise in repeated samples from women throughout pregnancy.[171,179] The values obtained for estriol-16α-glucosiduronate in plasma were two to three times those reported for estriol. This suggests that estriol-16α-glucosiduronate levels of plasma could be effectively used as a measure of fetal well-being.

Young et al.[126] developed a method for separating triethylammonium salts of estriol conjugates on Sephadex LH-20. The purified conjugates were then hydrolyzed, and the estriol was measured by radioimmunoassay. This method was first used to measure the estriol conjugates in amniotic fluid of normal and Rh-immunized patients. Estriol-3-sulfate ranged from 29 to 348 ng/mℓ in nine normal subjects at 32 to 40 weeks of gestation. The concentration of this conjugate was similar (16 to 225 ng/mℓ) in four Rh-immunized patients at 28 to 37 weeks of gestation. The concentration of estriol-3-

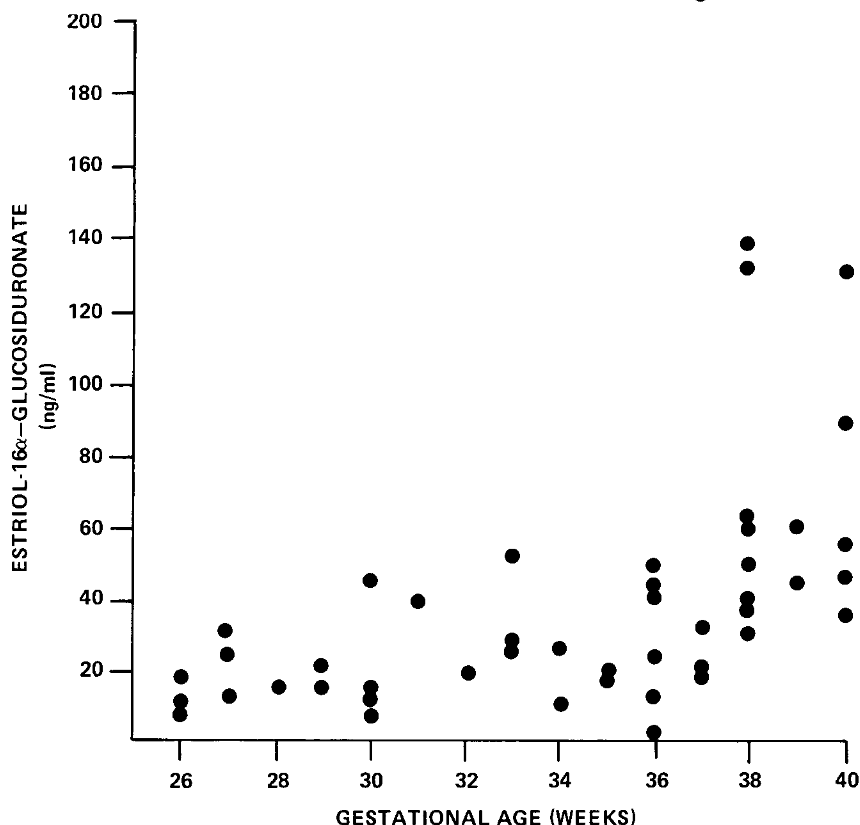

FIGURE 2. Plasma concentration of estriol-16α-glucosiduronate from 27 pregnant women from the 17th week of gestation to term.[174]

glucosiduronate ranged from 20 to 137 ng/mℓ in nine normal patients at 32 to 40 weeks of gestation and 27 to 247 ng/mℓ in four Rh-immunized patients at 28 to 37 weeks of gestation. No consistent relationships between these conjugates were evident in any patient. The authors concluded that the concentration of these conjugates did not reflect the progress of Rh disease.[126] In 14 normal pregnancies, the level of estriol-16α-glucosiduronate (151 to 585 ng/mℓ) increased with gestational age, while estriol-3-sulfate-16α-glucosiduronate remained constant or decreased (77 to 319 ng/mℓ). This resulted in an increased ratio of estriol-16α-glucosiduronate to estriol-3-sulfate,16α-glucosiduronate with gestation. However, in seven patients with Rh disease, not only did estriol-16α-glucosiduronate begin to decrease with advanced gestation, but estriol-3-sulfate, 16α-glucosiduronate increased, producing a decreasing ratio which was predictive of Rh disease.[126]

Estriol conjugates were measured simultaneously in plasma and urine in late pregnancy,[180,181] and the renal clearances were calculated for the four conjugates. Estriol-3-sulfate,16α-glucosiduronate was present in the highest concentration in the plasma, whereas estriol-16α-glucosiduronate was highest in the urine. The renal clearances of estriol-3-sulfate and of estriol-3-sulfate, 16α-glucosiduronate were less than that of inulin whereas that of estriol-16α-glucosiduronate was approximately equivalent to that of ϱ-aminohippuric acid. This finding was confirmed by Wright et al.,[174] who reported a mean renal clearance of 404 mℓ/min, which approximates the clearance of ϱ-aminohippuric acid and greatly exceeds that of inulin. These results suggest that estriol-16α-glucosiduronate is actively secreted by the renal tubules. However, the con-

tribution of the postulated renal production and direct excretion of estriol-16α-glucosiduronate is unknown.[91]

Raju et al.[182] measured the endogenous levels of four estriol conjugates — 3-sulfate, the 3- and 16α-monoglucosiduronates, and the sulfoglucosiduronate — in normal plasma and breast cyst fluid. The plasma concentration of each of the estriol conjugates was seldom detectable in eight normal women. However, four subjects had luteal phase levels of estriol-3-sulfate ranging from 20 to 76 pg/mℓ and one had plasma concentrations of estriol-16α-glucosiduronate and of estriol-3-glucosiduronate, respectively, of 132 and 253 pg/mℓ. In fluid aspirated from breast cysts to ten premenopausal women, the concentration of estriol-3-sulfate ranged from 240 to 4310 pg/mℓ, whereas the other three conjugates were undetectable in two patients and ranged from 13 to 153 ng/mℓ in eight patients. Only three patients had detectable amounts of estriol (30, 12, and 12 pg/mℓ). The high concentration of estriol-3-sulfate in breast cyst fluid is both surprising and unexplained. Unless there is preferred retention of this conjugate by cyst fluid, it probably does not originate from plasma sources.

Estriol-16-sulfate from human pregnancy fluids was purified by alumina, celite, and Sephadex LH-20 chromatography by Levitz et al.,[127] and the hydrolyzed estriol was measured by radioimmunoassay. Estriol-3,16-disulfate ranged from 0.4 to 3.0 ng/mℓ in maternal serum, from 6 to 28 ng/mℓ in cord serum, and from 2 to 7 ng/mℓ in amniotic fluid ($n = 4$). Estriol-16-sulfate was undetectable to 0.4 ng/mℓ in maternal serum, undetectable to 0.9 ng/mℓ in cord serum, and 0.5 to 8 ng/mℓ in amniotic fluid ($n = 4$). The contribution of both conjugates to total estriol was less than 1%.

Touchstone and Dobbins[131] acetylated and quantitated by gas-liquid chromatography estriol-3-sulfate and 16α-hydroxydehydroisoandrosterone-3-sulfate from ten samples of cord blood. The concentrations ranged from 120 to 660 ng/mℓ and from 10 to 2590 ng/mℓ, respectively.

Estrone sulfate has been measured following solvent partition by Brown and Smyth,[123] after solvolysis by Loriaux et al.[122,183] and Hawkins and Oakey,[184] and as the intact conjugate by Wright et al.[185] The data are summarized in Table 7. Hawkins and Oakey measured plasma estrone sulfate throughout two menstrual cycles and found considerable daily variation in the follicular levels in one subject and in the luteal levels in both subjects.[184]

Specific radioimmunoassays for urinary estrone glucosiduronate, 17β-estradiol-17-glucosiduronate and estriol-16α-glucosiduronate in normal men and women have been developed.[175,176] The urinary excretion of these conjugates is shown in Table 8. Of the conjugates measured, estrone glucosiduronate is excreted in the greatest amounts in both men and women. Estrone sulfate was measured in the urine of five normal men and five women at various times in the cycle and its excretion compared to that of estrone glucosiduronate. Estrone sulfate excretion ranged from 0.8 to 7.9 μg/24 hr in men and 5.1 to 18.7 μg/24 hr in women. The excretion of estrone glucosiduronate in these subjects was generally two to seven times that of estrone sulfate.[175,185]

The daily urinary excretion of estrone glucosiduronate, 17β-estradiol-17-glucosiduronate, and estriol-16α-glucosiduronate was measured throughout the menstrual cycle in seven normal women.[174,175] During the follicular phase, the mean daily excretion (μg/g creatinine) varied from approximately 13 to 30 for estrone glucosiduronate, 1.3 to 4.0 for 17β-estradiol-17-glucosiduronate, and 1.4 to 10 for estriol-16α-glucosiduronate. For all three glucosiduronates, a midcycle peak occurred with a mean value of 61.1 for estrone glucosiduronate, 19.4 for estriol-16α-glucosiduronate, and 8.2 for 17β-estradiol-17-glucosiduronate. The midcycle peak was followed by a lower luteal phase peak approximately 5 to 7 days after the midcycle peak.

Figure 3 shows the mean daily urinary output ± SE for estrone glucosiduronate (μg/g creatinine) throughout the menstrual cycle of seven women. The pattern of excretion was characterized by a low level during the follicular phase followed by a midcycle

TABLE 7

Plasma Concentration of Estrone Sulfate (ng/mℓ) in Normal Men and Women

Subject	n	Mean	Range	Ref.
Follicular phase		0.30	0.15—0.43	123
At ovulation		3.34	1.42—4.88	
Males	30	0.462	0.17—1.12	122
Follicular phase	19	0.654	0.14—1.40	
Luteal phase	31	1.247	0.28—2.80	
10 weeks gestation		4.0		183
35 weeks gestation		80.0		
Males	6	0.716	0.84—1.15	184
Males	13	0.922	0.46—1.23	185
Follicular phase	15	0.972	0.53—1.58	
Luteal phase	15	1.806	0.81—3.35	

peak. The levels of estrone glucosiduronate then fell to a low value followed by a second luteal phase peak. The mean daily excretions for 17β-estradiol-17-glucosiduronate and estriol-16α-glucosiduronate were considerably lower than those for estrone glucosiduronate, but were also characterized by a sharp midcycle peak and a lower luteal phase peak.

IV. METABOLISM OF STEROID CONJUGATES

The administration of conjugates labeled with ^{3}H in the steroid nucleus and ^{14}C or ^{35}S in the conjugating moiety has demonstrated that steroid conjugates may be metabolized extensively before excretion. The glycosidic or ester linkage is often preserved while the steroid moiety undergoes further metabolism. The mode of administration, oral or intravenous, may have significant effects on the ultimate fate of the conjugate. In addition, the enterohepatic circulation plays an important role in metabolic dynamics of steroid conjugates, particularly estrogen conjugates. This compartment may be responsible for variations with time in the excretion of exogenous steroid conjugates and their metabolites.

Hydrolysis of conjugates, a principal metabolic process, is mentioned briefly in this section, but is dealt with at greater length in Section V.

A. Glucosiduronates

1. Estriol Glucosiduronates

The 3- and 16α-glucosiduronates of estriol have been the most widely studied estrogen conjugates. Metabolic studies were facilitated by the early establishment of methods for the biosynthesis of radioactive estriol glucosiduronates.[2,11] Estriol glucosiduronates are of particular importance in pregnancy for monitoring fetal well-being. Much of our current knowledge about the formation, metabolism, and excretion of estriol and estriol conjugates in the major organs of the human is summarized in Figure 4.

Goebelsmann et al.[186,187] administered ^{14}C-estriol-16α-glucosiduronate to a pregnant woman. More than 86% of the radioactivity was excreted within 96 hr, of which 70% was estriol-16α-glucosiduronate and 16% was estriol-3-glucosiduronate. However, most of the 16α-glucosiduronate was excreted on the first day. On subsequent days, equal quantities of both conjugates were excreted. After the simultaneous intravenous infusion of ^{3}H-estriol-3-glucosiduronate and ^{14}C-estriol-16α-glucosiduronate, the major urinary form of both isotopes was estriol-3-glucosiduronate.[188] Small amounts of

TABLE 8

Mean Concentration ± SE (With Ranges in Parentheses) of Estrone Glucosiduronate, 17β-Estradiol-17-glucosiduronate, and Estriol-16α-glucosiduronate in the Urine of Men and Women in the Midfollicular (Day 8) and Midluteal (Day 22) Phases of the Menstrual Cycle Determined by Direct Radioimmunoassay (Values Expressed as μg/g Creatinine)[175]

	Follicular female mean ± SE (n) (range)	Luteal female mean ± SE (n) (range)	p for difference between follicular and luteal phase	Male mean ± SE (n) (range)	p for difference between male and follicular phase
Estrone glucosiduronate	17.3 ± 1.6 (18) (6.8—81.5)	31.8 ± 2.5 (15) (9.8—47.9)	< 0.01	10.1 ± 0.6 (6) (7.6—11.3)	< 0.05
17β-Estradiol-17-glucosiduronate	2.4 ± 0.1 (18) (0.9—3.4)	4.2 ± 0.4 (15) (2.2—7.5)	< 0.01	1.7 ± 0.3 (6) (1.0—2.9)	< 0.05
Estriol-16α-glucosiduronate	4.7 ± 0.9 (18) (1.3—16.5)	10.0 ± 1.6 (15) (3.2—23.4)	< 0.01	1.8 ± 0.2 (6) (1.0—2.5)	< 0.05

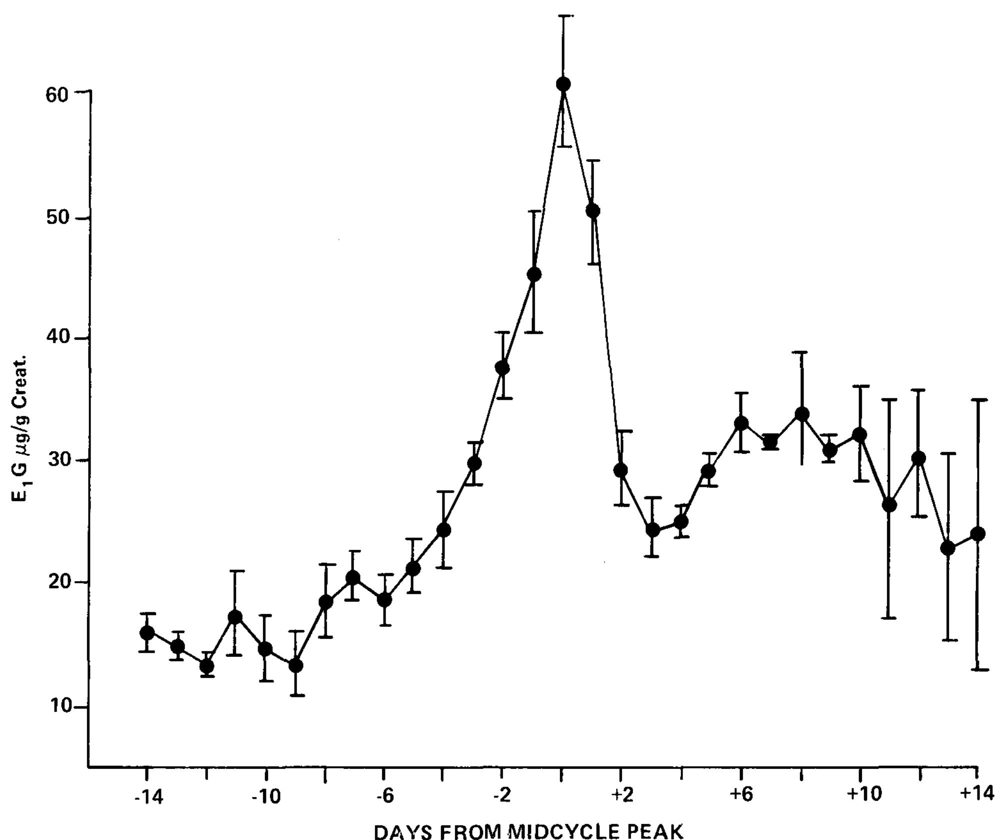

FIGURE 3. Mean urinary excretion of estrone glucosiduronate (E,G) (µg/g) creatinine throughout the menstrual cycles of seven women.[176]

^3H appeared as the 16α-glucosiduronate, suggesting that both estriol glucosiduronates undergo hydrolysis and reconjugation and that the 3-glucosiduronate is the favored product. However, Sandberg and Slaunwhite[189] found that nearly all estriol-16α-glucosiduronate was excreted unchanged in the urine following its intravenous administration to women. Kirdani et al.[190] found urinary $^3H/^{14}C$ unchanged in ^3H-estriol-3-^{14}C-glucosiduronate after injection of this compound. There is now considerable evidence that estriol-3-glucosiduronate is biosynthesized in the human intestine, but not in the liver.[12,192,193]

The biosynthesis of estriol-3,16α-diglucosiduronate has not been demonstrated in the human, although the rabbit can form this conjugate.[68] When Kirdani et al.[191] administered intravenously double-labeled estriol-3,16α-diglucosiduronate to humans, both glucosiduronate groups were rapidly hydrolyzed, and the phenolic hydroxyl group reconjugated with glucosiduronic acid. A small amount of the conjugate was excreted unchanged.

2. Estrone and Estradiol Glucosiduronates

Figure 5 presents our concept for the formation, metabolism, and excretion of estrone and estradiol glucosiduronates in the human. The major part of intravenously administered estrone glucosiduronate is rapidly excreted unchanged in the urine.[194,195] However, Zucconi et al.[194] demonstrated that in the presence of a viable fetus, estrone glucosiduronate could be converted directly to 17β-estradiol-3-glucosiduronate and 16α-hydroxyestrone-3-glucosiduronate without prior hydrolysis. Roy and

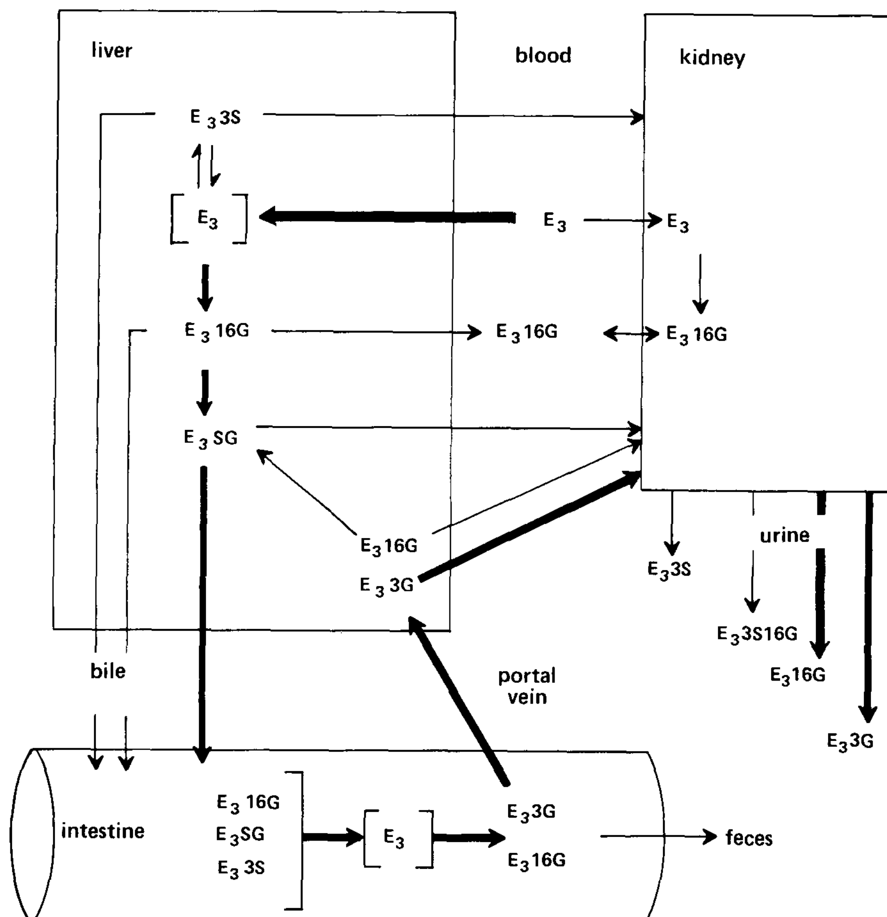

FIGURE 4. Major pathways for the metabolism of estriol conjugates.

Slaunwhite[196] demonstrated in vitro placental steroid dehydrogenases capable of interconverting estrone glucosiduronate and 17β-estradiol-3-glucosiduronate. As with the estrone and 17β-estradiol interconversion, the equilibrium favored the formation of estrone glucosiduronate. Hobkirk and Nilsen[195,197] reported in vivo interconversion of estrone glucosiduronate and 17β-estradiol-3-glucosiduronate in normal women after intravenous injection of the labeled compounds. Again, estrone glucosiduronate was the favored metabolite. This study also demonstrated a time dependent hydrolysis followed by reconjugation with endogenous glucosiduronic acid. Upon oral administration of ^{3}H-estrone glucosiduronate-^{14}C, however, little radioactivity was recovered in the double-labeled form, suggesting that the gastrointestinal tract is very efficient in the hydrolysis of estrogen glucosiduronates.[197]

The metabolism of 17β-estradiol-17-glucosiduronate also has been studied in women.[198,199] After intravenous injection, major urinary metabolites are estrone glucosiduronate and 17β-estradiol-3-glucosiduronate. Other metabolites include estriol glucosiduronates, 16-oxygenated estrone glucosiduronate, 2-methoxy-estrone glucosiduronate, estrone sulfate, and 17β-estradiol-3-sulfate,17-glucosiduronate, as well as the starting material.[198,199] Musey et al.[200] found the early metabolites (within 3 hr of intravenous injection) to be primarily 17β-estradiol-17-glucosiduronate and 17β-estradiol-

MAJOR PATHWAYS FOR METABOLISM OF ESTRONE
SULFATE, ESTRONE AND ESTRADIOL - 17β

FIGURE 5. Major pathways for metabolism of estrone and 17β-estradiol conjugates.

3-sulfate,17-glucosiduronate. Later metabolites were A-ring glucosiduronates. After oral administration, very little 17β-estradiol-17-glucosiduronate but mainly 3-glucosiduronates were recovered in the urine,[197] implicating the gastrointestinal tract in hydrolysis and reconjugation. Our concept for the formation, metabolism, and excretion of 17β-estradiol-17β-glucosiduronate in the human is shown in Figure 6.

B. Estrogen Sulfates

The metabolism of estrone sulfate is of particular interest because it is quantitatively the major form of plasma estrogen[63,201,202] in normal men and women. It is metabolized extensively in vivo,[203] and the principal biologically active hormones can be regenerated by a combination of hydrolysis (estrone) and reduction (17β-estradiol).[61,124] Like estrone glucosiduronate, estrone sulfate can be metabolized without prior hydrolysis of the sulfate group, but the sulfate linkage appears to be more labile than the glucosiduronate. Lewison et al.[205] found estrone sulfate to be hydrolyzed rapidly and the steroid nucleus to be excreted largely as β-glucuronidase-hydrolyzable material. Fishman and Hellman[203] found that increased amounts of urinary estrogen sulfates were excreted after administration of estrone sulfate and proposed that it, rather than estrone, was the major precursor of the more highly oxygenated estrogen metabolites. Estrone sulfate can also be metabolized and excreted in the urine as 17β-estradiol-glucosiduronates and estriol glucosiduronates.[206] When Emerman et al.[207] perfused human fetuses with 6,7-^{3}H-estrone-^{35}S-sulfate, several unidentified estrogen sulfates, as well as 17β-

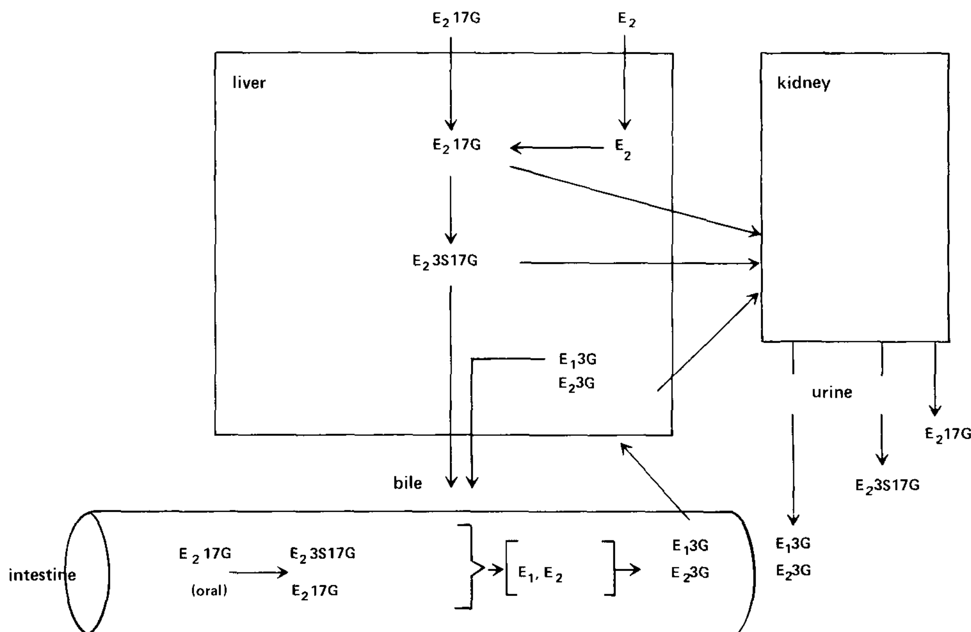

FIGURE 6. Major pathways for the metabolism of 17β-estradiol-17-glucosiduronate.

estradiol-3-sulfate, with the same $^{3}H/^{35}S$ ratio as the perfused estrone sulfate were found in the liver. This suggests that small amounts of estrone sulfate may also be metabolized directly to 17β-estradiol-3-sulfate without prior hydrolysis of the sulfate group.

Similar results were not seen when double-labeled estriol sulfate was infused into adult subjects.[208] More than 75% of the injected tritium was collected in the bile as estriol-3-sulfate,16α-glucosiduronate without ^{35}S, implying hydrolysis of the sulfate group followed by reconjugation of the estriol with both the sulfuric acid and glucosiduronic acid radicals. Further confirmation was provided by Oertel et al.,[209] who found the sulfoconjugated estrogens in plasma had a $^{3}H/^{35}S$ ratio similar to the injected compound, whereas the urinary ratio was increased. In studies where double-labeled estrone sulfate was injected, minute quantities of other estrogen sulfates with $^{3}H/^{35}S$ ratios similar to the injected material were isolated in both urine and bile in the early period after injection.

The isolation of N-acetylglucosamine conjugates of estrogens from human bile was first reported by Jirku and Levitz[210] in their investigation of the metabolism of estrone sulfate. This conjugate accounted for 24% of the injected steroid recovered in the bile and was identified as the sulfo-N-acetylglucosaminide form of 15α-hydroxyestrone and 15α-hydroxyestradiol-17β. Conjugates of N-acetylglucosamine have been shown to be major estrogen metabolites in the rabbit.[30,67] The N-acetylglucosamine is linked to the steroid primarily at the 15α position in the human, while in the rabbit conjugation occurred at the 17α position.

C. Estrogen Sulfoglucosiduronates

Estrogen sulfoglucosiduronates are the major form of double conjugates in the human.[63,65,211] The sulfoglucosiduronates are also the primary steroid conjugates secreted

into the bile. (See later section on enterohepatic circulation.) Formation of double conjugate of estriol proceeds through the steps: estriol → estriol-16α-glucosiduronate → estriol-3-sulfate, 16α-glucosiduronate.[208,212] Considerable evidence exists that suggests a similar pathway exists for the double conjugate of 17β-estradiol; 17β-estradiol → 17β-estradiol-17-glucosiduronate → 17β-estradiol-3-sulfate,17-glucosiduronate.[200]

The metabolism of estriol-3-sulfate,16α-glucosiduronate has been studied after administration of this compound intraduodenally and intravenously.[213] After intraduodenal administration, none of the sulfoglucosiduronate was excreted in the urine. However, following intravenous administration, estriol-3-sulfate,16α-glucosiduronate was a major urinary metabolite in the first hour. The levels declined thereafter. These data indicate that estriol-3-sulfate,16α-glucosiduronate is excreted in the urine if it is present in the peripheral circulation, but, after entry into the enterohepatic circulation, it is rapidly metabolized. The primary metabolite after biliary secretion and intestinal reabsorption is estriol-3-glucosiduronate.[213]

Estriol and 17β-estradiol monosulfates have not been found as metabolites of the sulfoglucosiduronate in either bile or urine, indicating that estrogen sulfoglucosiduronates undergo hydrolysis of both conjugating moieties in the small intestine. Subsequent reconjugation with glucosiduronic acid occurs primarily at the phenolic hydroxyl group. Some conjugation at the 16α position also occurs.

D. Other Estrogen Conjugates

A number of investigators have conducted studies involving some novel estrogen conjugates in both humans and animals. In general, the metabolism of these conjugates is similar to that of the classical conjugates considered above. Estrogen glucosides and N-acetylglucosaminides were found to be the primary urinary estrogen conjugates of the rabbit.[30,67,214] In addition, minor quantities of the latter have been isolated recently from human bile.[210]

The metabolism of estrogen-3-glucoside, unlike that of the 3-glucosiduronate, is characterized by rapid hydrolysis in both the human and rabbit. The fate of 17β-estradiol-17-glucoside in the human is similar to that of 17β-estradiol-17-glucosiduronate.[200,215] Although no biliary studies have been conducted, the time course for the appearance of metabolites of 17β-estradiol-17-glucoside in the urine suggests a rapid 3-glucuronidation to produce the double conjugate, which is preferentially excreted into the bile.[215] Thereafter, the double conjugate undergoes hydrolysis followed by reconjugation in the intestinal tract and subsequent excretion as the 3-glucosiduronate in the urine.

E. Neutral Steroid Conjugates

There is considerable evidence in the literature indicating that both sulfates and glucosiduronates of testosterone, dehydroisoandrosterone, and corticosterone, as well as other neutral steroids, can be metabolized without prior hydrolysis. These neutral steroid conjugates may also undergo hydrolysis followed by reconjugation as discussed above for estrogen conjugates. The metabolism of the neutral steroid conjugates has been reviewed.[216,217]

Cholesterol sulfate and dehydroisoandrosterone sulfate deserve special mention, because they are secreted as well as formed peripherally. Lieberman et al.[218] provided most of the experimental data to show that the integrity of the ester linkage of cholesterol sulfate may be preserved while metabolism is occurring. Following intravenous administration of ^{3}H-cholesterol-^{35}S-sulfate to a woman with carcinoma of the adrenal cortex, 5-pregnene-3β,17β,20α-triol-3-sulfate, dehydroisoandrosterone sulfate, 5-androstene-3β,17β-diol-3-sulfate, and 5-androstene-3β,16α-diol-17-one-3-sulfate with $^{3}H/^{35}S$ ratios similar to the injected material were isolated from the urine.[218-220] In

contrast, Gurpide et al.[221] were unable to show that ^{14}C-cholesterol sulfate could be converted by the adrenal to cortisol sulfate in women with adrenal carcinoma or to dehydroisoandrosterone sulfate in normal women and suggested that plasma cholesterol sulfate is a minor precursor of the adrenal steroid sulfates.

Siiteri and MacDonald[222] have shown that intravenously administered dehydroisoandrosterone sulfate is converted to estrogens in high yield by pregnant women. These findings have been confirmed by Bolte et al.[223] and others[224-227] regardless of the route of administration (oral or intravenous).[227] In contrast, oral administration of dehydroisoandrosterone glucosiduronate to a normal male resulted in the urinary excretion of only androsterone glucosiduronate and etiocholanolone glucosiduronate.[228]

F. Enterohepatic Circulation

Many steroids are conjugated by the liver and subsequently excreted into the small intestine via the bile. In the gut, these steroid conjugates are hydrolyzed by the bacteria, reabsorbed by the intestinal wall, and reconjugated. The reconjugated steroid is transported back to the liver via the portal vein. This phenomenon was first described in dogs by Cantarow et al.[229] using large doses of estrone. The enterohepatic circulation of estrogens has been described for a number of different species,[230] including the human.[231,232]

The enterohepatic circulation described above suggests a closed circuit where the steroid conjugates are circulated almost indefinitely, like the bile acids.[233] This is not the case for most steroids such as estrogens, because about 50% of the injected steroid is excreted in the urine within 24 hr. Evidence reported by several groups[232,234,235] suggests the hepatocytes may play a role in this process by sequestering certain types of steroid conjugates for subsequent release into the blood where they are quickly excreted by the kidney. Double conjugates of estrogens, such as estriol-3-sulfate,16α-glucosiduronate and 17β-estradiol-3-sulfate,17-glucosiduronate are preferentially excreted in the bile,[200,208,213] while monoglucosiduronates such as estrone glucosiduronate and estriol-3-glucosiduronate are released into the blood and excreted primarily in the urine.[208,210]

Molecular structure and size influence the biliary secretion[236,237] of steroid conjugates. For example, estriol-16α-glucosiduronate and 17β-estradiol-3-glucosiduronate are excreted in the bile, whereas estriol-3-glucosiduronate and 17β-estradiol-17-glucosiduronate are excluded from the bile.[200] As a general rule, the more polar the estrogen conjugate, the more likely it is to be excreted into the bile. Thus, double conjugates tend to appear in bile more than monosulfates, which in turn exceed the monoglucosiduronates. Unconjugated steroids are generally not excreted in the bile.

A number of investigators have contributed to our understanding of the enterohepatic circulation of estriol in the human.[186,187,189,200,208,210,231,238,239] These interrelationships are outlined in Figure 4. The liver converts estriol to the monoconjugates, estriol-3-sulfate and estriol-16α-glucosiduronate, and the double conjugate, estriol-3-sulfate,16α-glucosiduronate, all of which are excreted in both urine and bile. The conjugates excreted in the bile undergo hydrolysis in the gut to yield free estriol which is reabsorbed into the intestinal wall and reconjugated with glucosiduronic acid at the 3- or 16α- position. The estriol-3-glucosiduronate is quantitatively excreted into the urine while the estriol-16α-glucosiduronate is, in part, converted to the double conjugate, estriol-3-sulfate, 16α-glucosiduronate in the liver and excreted again into the bile.

Steroid conjugates excreted into the bile show a low and prolonged urinary excretion.[189,208,231,238] Our understanding of the role of the enterohepatic circulation of estrogens in humans comes primarily from a series of reports from the laboratories of Sandberg and Slaunwhite[189,231] and Levitz et al.[208,238,239] Sandberg and Slaunwhite reported that intravenously administered estriol-16α-glucosiduronate was excreted intact in the

urine with no biliary excretion.[189] These results contrast with the findings of Goebelsmann et al.,[186] who reported that 20% of intravenously administered ^{14}C-estriol-16α-glucosiduronate was excreted in urine as estriol-3-glucosiduronate. Estriol-16α-glucosiduronate predominated during the first day after injection, but about equal quantities of both 16α- and 3-glucosiduronate appeared during subsequent days. This discrepancy was clarified by Levitz et al.,[239] who injected radioactive estriol-16α-glucosiduronate into human cholecystectomy subjects with T-tube drainage and found a significant amount of radioactivity (28%) in the bile. The bile contained mostly estriol-3-sulfate, 16α-glucosiduronate with small but significant amounts of estriol-16α-glucosiduronate. The converse was true for the urine, but no estriol-3-glucosiduronate was identified. In intact subjects, estriol-3-glucosiduronate was derived almost exclusively from biliary estriol-3-sulfate, 16α-glucosiduronate and estriol-16α-glucosiduronate.[239]

Information about the metabolism, conjugation, and enterohepatic circulation of estrone and 17β-estradiol and their conjugates in the human is shown in Figure 5. Hobkirk and his co-workers[197-200,240,241] have conducted a number of studies on the metabolism and enterohepatic circulation of estrone and 17β-estradiol and their conjugates in the human. When radioactive 17β-estradiol was injected, estrone sulfate was on occasion the major metabolite in urine, although both sulfates and glucosiduronates of 17β-estradiol, 2-methoxyestrone, estrone, and the 16-oxygenated derivatives of 17β-estradiol were present.[240] In another study, 17β-estradiol-17-glucosiduronate was the sole metabolite for the first 10 min after injection, leading the authors to suggest that 17β-estradiol-17-glucosiduronate is a direct and perhaps unique metabolite of peripherally injected 17β-estradiol in the human.[241,242]

Studies of the metabolism of radioactive 17β-estradiol-17-glucosiduronate in normal women showed that most of the urinary metabolites (80%) were estrone- and 17β-estradiol glucosiduronates in the ratio of 1:1 with only small amounts of 16-oxygenated metabolites as well as estrone sulfate and estradiol-3-sulfate,17-glucosiduronate.[242] These reports suggested that the metabolism and enterohepatic circulation of 17β-estradiol-17-glucosiduronate was similar to that of estriol-16α-glucosiduronate (Figure 6). 17β-Estradiol is transported to the liver and/or kidney where 17β-estradiol-17-glucosiduronate is formed and then efficiently sulfurylated at the phenolic hydroxyl group to form the double conjugate. Like the estriol sulfoglucosiduronate, estradiol-3-sulfate,17-glucosiduronate is primarily excreted via the bile (85 to 97%) into the small intestine where hydrolysis of both the glucosiduronate and sulfate groups occurs.[200] Hydrolysis is followed by reabsorption of the free estrogen into the mucosa of the intestinal tissue and reconjugation of the phenolic hydroxyl group to form estrone glucosiduronate and 17β-estradiol-3-glucosiduronate.[200] These monoglucosiduronates are carried to the liver via the portal system and subsequently excreted principally in the urine.[195,200] In addition, Hobkirk and co-workers obtained evidence for an as yet unidentified sulfoglucosiduronate metabolite that is excreted predominantly in the bile with less than 1% appearing in the urine.[200]

V. HYDROLYSIS OF STEROID CONJUGATES

Considerable evidence has been presented in the foregoing section to indicate that steroid conjugates are metabolized extensively in vivo. One of the major metabolic steps is hydrolysis of the conjugating group to give the free steroid. Most of the information on the hydrolysis of steroid conjugates was obtained from in vitro experiments, and these have been reviewed by a number of workers.[243-245] The present review will concentrate on the data obtained from in vivo experiments.

A major site of metabolic hydrolysis of steroid conjugates is the duodenal region of the intestinal tract. Several workers have shown that steroid conjugates brought to the

gut via the bile undergo almost total hydrolysis followed by subsequent reconjugation.[197,200,205,208,213,246,247] This hydrolysis is thought to be primarily due to enzymes present in the intestinal bacteria rather than the intestinal tissue. However, Lisboa et al.[248] obtained evidence that the tissue of the intestinal wall might be involved directly in the hydrolysis of estrogen sulfates when they incubated 17β-estradiol-3-sulfate in sacs of everted small intestine and showed that it was hydrolyzed to free estrone and estradiol-17β as well as converted to their glucosiduronates.

The hydrolysis of estrogen conjugates present in the systemic circulation has been studied.[56,59] Such hydrolysis is of importance because of its potential as a source of circulating physiologically active estrogens. In vivo hydrolysis of administered ^{3}H-estrone sulfate has been demonstrated in the human by at least two different research groups.[54,55] Ruder et al.[54] reported a conversion ratio for estrone sulfate to estrone ($CR_{ES \rightarrow E1}$) in humans of 0.008 to 0.025 and a transfer constant from estrone sulfate to estrone ($\varrho_{ES \rightarrow E1}$) of 0.13 to 0.30. The latter values, which indicate that 13 to 30% of the total production of estrone sulfate is hydrolyzed to estrone, were confirmed by Longcope.[55]

The dynamics of estrone glucosiduronate have been studied in the dog.[56] The mean conversion ratio for estrone glucosiduronate to estrone was 0.021. The mean transfer constant for estrone glucosiduronate to estrone (ϱ_{EG-E1}) was 0.057, indicating that approximately 6% of the total amount of estrone glucosiduronate produced is converted to estrone.

Considerable in vitro data[246,259] suggest that both sulfatase and β-glucuronidase enzymes are present in the liver. Yet there is no good evidence for in vivo hydrolysis of steroid conjugates by the liver or other tissues in the human. For example, when radioactive estriol-16α-glucosiduronate was administered directly into the portal system, no evidence was obtained for hydrolysis by the liver.[246] This could result from the complexity of the patterns of biosynthesis and metabolism that are occurring simultaneously in the liver.

The only direct evidence for the in vivo hydrolysis of steroid conjugates other than estrone sulfate comes from a series of studies in the dog by Preedy and coworkers.[56,59,259] The protocol used in these experiments was to infuse radioactive estrogens or estrogen conjugates under steady-state conditions and collect simultaneous blood samples from various sites draining one organ or area. When either radioactive estrone or estrone glucosiduronate was infused, the mean percent extractions of total unconjugated estrogens as well as unconjugated estrone and 17β- estradiol by the lungs were negative while those of estrone glucosiduronate and 17β-estradiol glucosiduronates were positive. This suggests that the lungs are an important site for hydrolysis of estrogen glucosiduronates.[59] Collins et al. obtained simultaneous plasma samples from the right ventricle and brachial artery of eight patient volunteers undergoing cardiac catheterization for diagnostic purposes.[262] The endogenous levels of both estrone and 17β-estradiol were significantly higher in the brachial artery, suggesting that the lungs are an active site for production of free estrogens in the human (presumably by hydrolysis of estrogen conjugates). The large blood flow through the lung makes even a small rate of hydrolysis significant and important as a source of free estrone and 17β-estradiol.

A small but significant uptake of estrone glucosiduronate (12.0 ± 3.7%) was shown for the spleen;[255] this most likely reflects hydrolysis of small quantities of estrone glucosiduronate. This organ is known to contain substantial amounts of β-glucuronidase activity in various animal species. Another organ showing a substantial uptake of estrone glucosiduronate (16%) is the hind limb of the dog.[249] However, there was also a substantial uptake of total radioactivity, indicating some retention unchanged in the area or removal by routes other than the femoral vein. Evidence that the radioactivity may be removed via the lymphatic system was also presented.[249]

The placenta is active in the hydrolysis of steroid sulfates.[207,250-253] Essentially all steroid sulfates present in the placenta are quantitatively hydrolyzed and subsequently released as free steroids. Unlike the dog lung, which hydrolyzes estrogen glucosiduronates but not sulfates, the placenta hydrolyzes steroid sulfates, but not glucosiduronates.

VI. REGIONAL FORMATION AND METABOLISM OF ESTROGEN CONJUGATES

The regional formation and metabolism of steroid conjugates in the dog have been studied by Preedy and co-workers.[56,59,249,254-259] The dog was used as a model because of the relative inaccessibility of the veins draining the various sites and organs in the human. The metabolism of estrogens in the dog is similar to the human in most,[255] but not all,[254] respects. A basic experimental design was used which involved the constant-rate infusion of unconjugated or conjugated estrogens (principally ^{3}H-estrone, ^{3}H-estriol, and ^{3}H-estrone glucosiduronate), followed by measurement of the infused estrogen and its metabolites simultaneously in arterial plasma (A) and in plasma from the appropriate efferent vein (V).[255,256] From these data arterio-venous concentration differences (A-V) and the percent extractions [100 (1 - V/A)] were determined across various organs (i.e., hind limb, lungs, etc.) and various areas (e.g., splanchnic area). A similar technique was used by Chapdelaine to study the regional metabolism of testosterone and Δ_4-androstenedione, using different labels to enable measurement of interconversions between these hormones.[260] Longcope et al.[261] used a similar double-label technique to study estrogen metabolism in the human forearm.

Preedy and co-workers[255-259] used a single-labeled technique, which has the restriction that interconversion between two hormones cannot be quantitated. However, it is a simpler procedure and retains the ability to measure simultaneously both arterio-venous differences and percent extractions of the infused radioactive steroid and any number of radioactive metabolites across an organ or area. Positive values for the percent extraction indicate net uptake by the tissue or area, and negative values indicate net formation for release into the venous effluent. Uptake by the tissue may reflect metabolism, excretion, or removal by lymphatics, or all three. It is theoretically possible that uptake without metabolism or removal could occur, but this seems unlikely. In addition, since only net effects are measured, uptake and formation can occur simultaneously.

In a series of experiments carried out by Collins et al.,[255] ^{3}H-estrone was infused, and the simultaneous percent extractions of ^{3}H-estrone and of its radioactive metabolites 17β-estradiol, estrone sulfate, estrone glucosiduronate, and 17β-estradiol glucosiduronate(s) across the splanchnic bed and the intestine were determined. The results are summarized in Table 9.

The mean percent extractions $\pm$ SE of the unconjugated estrogens estrone and 17β-estradiol, across the splanchnic area were 86 $\pm$ 2 and 88 $\pm$ 3, respectively, indicating nearly complete extraction in one passage through the area. There was a positive percent extraction of estrone sulfate by the splanchnic area, but this was much smaller than for the unconjugated estrogens (28 $\pm$ 5). In contrast, the mean percent extractions of the conjugates, estrone glucosiduronate and 17β-estradiol glucosiduronate(s), were *negative,* being -49 ± 9 and -33 ± 8, respectively, indicating net formation of these conjugates within the splanchnic area for release into the venous effluent. The positive extraction of estrone sulfate could reflect either metabolism or excretion or both, since estrone sulfate is known to be present in substantial amounts in bile.

The splanchnic area comprises several major organs, namely, liver, intestine, spleen, and pancreas, any of which could be responsible for the splanchnic extractions noted

TABLE 9

Regional Extraction of Unconjugated and Conjugated Estrogens in the Dog

	Percent extraction ± SE by				
Estrogen	Splanchnic area	Intestine	Spleen	Lungs	Hind limb
Estrone	86 ± 2	45 ± 3	35 ± 4	−21 ± 4	40 ± 5
17β-Estradiol	88 ± 3	46 ± 13		−34 ± 6	32 ± 3
Estriol	77 ± 1	12 ± 4		NS[a]	22 ± 6
Estrone sulfate	28 ± 5	NS			−12 ± 4
Estrone glucosiduronate	−49 ± 9	−31 ± 8	12 ± 4	11 ± 2	16 ± 3
17β-Estradiol glucosiduronate(s)	−33 ± 8	NS		10 ± 2	
Estriol sulfate	−35 ± 8	−33 ± 10			
Estriol-3-glucosiduronate	−15 ± 3	−15 ± 7			
Estriol-16α-glucosiduronate	−23 ± 6	NS			
Estriol-3-sulfate, 16 α-glucosiduronate	NS	−53 ± 13			

Note: Values are mean ± SE for 5 to 10 dogs. Percent extraction = 100 (1 − V/A) where V and A are venous ad arterial plasma concentrations, respectively.

[a] No significant extraction.

above. To determine the role of the intestine, the constant infusion of ^{3}H-estrone was repeated, but with simultaneous sampling of artery and superior mesenteric vein to calculate the intestinal extraction.[255] The mean percent extractions ± SD of estrone and 17β-estradiol by the intestine were 45 ± 3 and 46 ± 3, respectively, indicating net uptake. In contrast, there were a negative extraction of estrone glucosiduronate −31 ± 8, indicating net formation of this conjugate by the intestine. The intestinal extractions of estrone sulfate and 17β-estradiol glucosiduronate(s) were not significantly different from zero.

The positive extraction of the unconjugated estrogens estrone and 17β-estradiol, by the intestine is most likely due to metabolism and the net formation of estrone glucosiduronate by the intestine. Net excretion in the feces via the intestinal lumen cannot be rigidly excluded, but this appears unlikely. There was a positive mean percent extraction of both estrone and estrone glucosiduronate by the spleen (35 ± 4 and 12 ± 4, respectively), indicating net uptake of both an unconjugated and conjugated estrogen by this organ, although the uptake of the glucosiduronate was relatively small.

From these studies we have an indication both of the overall activity of the splanchnic area in the extraction of unconjugated and conjugated estrogens, as well as the separate activities of the intestine and spleen. Although the liver was not studied directly, some information regarding hepatic activity can be inferred. Since the positive extraction of the unconjugated estrogens, estrone and 17β-estradiol, by the splanchnic area was approximately 90%, while the intestinal extraction was only approximately 45% and 35%, respectively, then positive extraction of these two estrogens by the liver must be approximately 45%. The situation with regard to estrone glucosiduronate is more complex. There is uptake by the spleen and net formation by the intestine and by the splanchnic area as a whole. Portal vein blood contains less estrone glucosiduronate than superior mesenteric vein blood (diluted by splenic vein blood), and hepatic artery blood contains less than either.[256,259] These data suggest that there is significant formation of estrone glucosiduronate by the liver as well as by the intestine.

Biliary excretion of estrone glucosiduronate is presumed to occur in the dog as well as in the human, and splanchnic extraction should reflect this. In addition, metabolism of estrone glucosiduronate might also be expected to occur. These processes would both lead to a *positive* extraction. However, this is, in fact, compatible with the observed negative extraction of estrone glucosiduronate by the splanchnic area, since, as already pointed out, all extractions are net effects. It is merely necessary to postulate that formation for release into the hepatic vein is greater than the sum of excretion plus metabolism.

Although it is not possible to distinguish between metabolism and excretion of estrone and 17β-estradiol by the splanchnic area in these experiments, the presence of one or both of these processes was easily demonstrated for estrone glucosiduronate by infusion of ^{3}H-estrone glucosiduronate and comparing the results to those obtained after infusion of ^{3}H-estrone. Since there is no *de novo* precursor of infused ^{3}H-estrone glucosiduronate, *de novo* formation by the splanchnic area is, by definition, eliminated. When this experiment was carried out,[259] the splanchnic extraction of estrone glucosiduronate was *positive* in contrast to a negative percent extraction after infusion of ^{3}H-estrone. These results indicate that metabolism or excretion or both is occurring simultaneously with formation of estrone glucosiduronate, but that formation predominates, as postulated.

Sites for hydrolysis of estrone glucosiduronate are important because of their potential as a source of physiologically active estrogens. Collins et al.[59] showed that the lungs hydrolyze estrone glucosiduronate in the dog and possibly in man. The percent extractions of conjugated and unconjugated estrogens across the lungs are given in Table 9.[59] There was net uptake of estrone glucosiduronate and 17β-estradiol glucosiduronate(s) (mean percent extraction ± SE = 11 ± 2 and 10 ± 2, respectively) and net formation of total unconjugated estrogens and of estrone and 17β-estradiol (−25 ± 5, −21 ± 4, and −34 ± 6, respectively), suggesting that the lung is a site of hydrolysis of estrogen glucosiduronates.

As mentioned previously, Longcope et al.[261] found a substantial uptake of estrone and 17β-estradiol in the human forearm and attributed it to metabolism. There was also evidence for the formation of estrone sulfate. Percent extractions by the hind limb of the dog were determined by Collins et al.[249] following an infusion of ^{3}H-estrone. The results are given in Table 9. In accordance with the findings of Longcope et al.[261] in the human forearm, substantial net uptake of estrone and 17β-estradiol was found (mean percent extraction ± SE = 40 ± 5 and 32 ± 3, respectively). There was also net uptake of estrone glucosiduronate (16 ± 3) and net formation of estrone sulfate (−12 ± 4), presumably from estrone, again in general agreement with the forearm results. An unexpected finding, however, was the substantial uptake of total radioactivity (mean percent extraction ± SE 31 ± 4) which cannot be explained by metabolism. Collins et al.[249] postulated that the radioactivity is removed from the hind limb by routes other than the efferent vein, such as the lymphatics. Accordingly, the levels of radioactive estrogens in the thoracic duct lymph during constant infusion of ^{3}H-estrone were estimated and compared with simultaneous arterial values. Total radioactivity in the thoracic duct reached 50% of the concentration of the arterial plasma in about 80 min, and the levels of unconjugated radioactivity were even higher (70%). Radioactive estrone, estrone sulfate, estrone glucosiduronate, and 17β-estradiol glucosiduronate(s) were all found in the lymph in substantial quantities, suggesting the lymph is active in the transport of estrogens.

Constant infusion of estriol was carried out under conditions similar to those described above,[257] and the percent extractions determined for estriol and its metabolites estriol-3-glucosiduronate, estriol-16α-glucosiduronate, and estriol-3-sulfate by the splanchnic region and intestine (Table 9). The mean splanchnic extraction ± SE of estriol was 77 ± 1, indicating net uptake similar to that of estrone and 17β-estradiol.

The mean splanchnic extraction of each of the three conjugates was negative, indicating net production of these conjugates by the splanchnic area, presumably by conjugation of estriol. The percent extraction of estriol by the intestine was positive (12 ± 4), indicating net uptake. The percent extraction of the three individual estriol conjugates by the intestine was negative, indicating net formation of these conjugates.[257] There was no significant extraction of estriol by the lungs.[59] However, substantial positive extraction of estriol by the hind limb was found (22 ± 6), indicating net uptake.[249]

In summary, the unconjugated estrogens, estrone, 17β-estradiol, and estriol, are taken up by the splanchnic area,[255,259] intestine,[255,259] spleen (estrone only studied),[255] and hind limb.[249] In contrast, estrone and 17β-estradiol (but not estriol) are formed in the lungs for release into the pulmonary vein, presumably through hydrolysis of estrogen conjugates.[59] The conjugates, estrone glucosiduronate, 17β-estradiol glucosiduronate(s), estriol sulfate, estriol-3-glucosiduronate, estriol-16α-glucosiduronate, and estriol-3-sulfate, 16α-glucosiduronate, are formed in the splanchnic area as a whole and in the intestine.[255,259] There is net uptake of estrone glucosiduronate by the spleen,[56] lungs,[59] and hind limb,[259] and of 17β-estradiol glucosiduronate(s) by the lungs,[59] all of which probably reflects metabolism including hydrolysis. The formation and metabolism of estrone sulfate appear to be somewhat different from the other estrogen conjugates. Net uptake by the splanchnic area and formation by the limbs have been reported in both the dog[249] and human[261] (Table 9).

VII. FACTORS THAT AFFECT STEROID CONJUGATION

The metabolism of steroid conjugates is altered by disease states,[224,263-272] drugs,[134,273] and even diet.[274-278] Since a significant amount of the conjugation and metabolism of steroids and steroid conjugates takes place in the liver, disease states which affect the liver, such as cirrhosis, result in significant alterations. The effect of liver disease on the metabolism and conjugation of estrogens has been reviewed.[233] Considerable controversy exists regarding the effect of liver cirrhosis on the plasma level of estrogens. Some groups have demonstrated that 17β-estradiol[263-269] and estrone[266] are higher than normal in cirrhotics, whereas other groups[279,280] could not demonstrate any significant differences. Since none of these groups classified the stage of cirrhosis, these results may reflect differences in the severity of the disease in the various groups of patients. Significant effects on the metabolism of estrogens in cirrhotics have been demonstrated by radioactive studies. Following intravenous administration of radioactive 17β-estradiol, more radioactivity was excreted in the urine of cirrhotics (71%) than normal men (51%).[269]

The excretion of steroid conjugates is severely altered in total extrahepatic biliary obstruction because normal biliary excretion is blocked. This leads to increases in urinary excretion of those steroid conjugates which would normally enter the gastrointestinal tract. Hellman et al.[272] found a significant increase in the urinary excretion of radioactivity within the first 24 hr of injection of ^{3}H-17β-estradiol in males and females with total biliary obstruction (48%) when compared to normal subjects (34%). The total recovery of radioactivity from patients with biliary obstruction was 70% compared to 57% in normal subjects. The inability of the normal biliary products to be excreted into the gut also resulted in an altered pattern of metabolism. The nonglucosiduronate fraction was 21% of the urinary radioactivity in patients with biliary obstruction compared to 12% in normal subjects. Significant differences were also noted in the percent of radioactivity of various individual estrogen metabolites. Estriol levels in urine of patients with biliary obstruction were about half that in the normal, whereas the levels of 16α-hydroxyestrone and estrone were about three times that in the normal subject.[253]

The effect of biliary obstruction on the metabolism of estriol is especially impressive.

Estriol-3-glucosiduronate is normally formed in the intestine after biliary excretion of estriol-16α-glucosiduronate and estriol-3-sulfate,16α-glucosiduronate where hydrolysis and reconjugation at the 3-position take place. The presence of biliary obstruction could lead to a significant increase in the urinary excretion of estriol-16α-glucosiduronate and estriol-3-sulfate,16α-glucosiduronate and a concomitant decrease in the urinary excretion of estriol-3-glucosiduronate.

Obesity,[271] hypothyroidism,[269,270,281] and breast cancer[271,273] have been reported to lead to increased D-ring metabolites (estriols) and decreased A-ring metabolites (2-hydroxyestrogens).[269] For example, Zumoff et al.[269] showed a decreased percent of radioactivity present in the urine as 2-hydroxyestrone and 2-methoxyestrone and an increased amount of 16α-hydroxylation in hypothyroidism and male breast cancer. On the other hand, hyperthyroidism leads to increased urinary excretion of estrogen as A-ring metabolites.[270] The consequence of these two competitive hydroxylations may have significant effects on the levels of estrogenic activity in plasma.[270] Both estriol and 2-hydroxyestradiol-17β have been shown to bind estrogen receptors effectively. The pattern of hydroxylation also has a significant effect on the patterns of conjugation of the estrogens. The predominant conjugation of the 16α-hydroxyl group is with glucosiduronic acid, whereas the 2-hydroxy metabolites are conjugated primarily with sulfuric acid.

Collins et al. studied the action on plasma estrogens of Sulfobromophthalein (BSP), a substance much used as a test of hepatic function in man.[256] BSP is excreted principally into the bile, and there is evidence that estrogens interfere with this process.[283] In a large number of dog experiments similar to those described above, BSP had a marked and sustained effect in raising the arterial plasma levels of the conjugates, estrone glucosiduronate, 17β-estradiol-glucosiduronate(s), and estrone sulfate, while leaving the concentration of estrone and 17β-estradiol unchanged, at least during the relatively short period of study.

Investigation of the mechanism of this striking effect revealed that BSP did not interfere with hepatic excretion or urinary excretion of these conjugates as was expected, nor did it alter the rate of formation (at least of estrone glucosiduronate). Consequently, the only remaining mechanism was a decrease in the rate of metabolism. One (and probably the principal)step involved in the metabolism of the glucosiduronates is hydrolysis, and a BSP-induced decrease in the rate of hydrolysis appeared to occur both in the lungs and hind limb. Thus, it appears that we have an agent which specifically alters the plasma level of estrogen conjugates. Whether unconjugated estrogen levels would eventually alter too, and whether there would be any therapeutic use for such a maneuver, remains unknown.

Evidence for relationships between breast cancer, estrogens, and diet has led to increased concern about the role of the intestinal microflora on the biosynthesis and metabolism of steroid conjugates. Much of the evidence for this activity by the intestinal microflora has been indirectly obtained from the oral administration of antibiotics.[273] This treatment results in drastic quantitative and qualitative changes in the intestinal microflora[273] as well as a reduction in the enteric hydrolysis and reconjugation of biliary steroid conjugates and epimerization and oxidation of steroids.[284-286] Engel[273] demonstrated that antibiotics reduced the percent of radioactive 17β-estradiol excreted in the urine. Adlercreutz et al.[133,287,288] later showed that giving ampicillin to pregnant women reduced the urinary excretion of endogenous estriol, 16α-hydroxyestrone, 16β-hydroxyestrone, 16-oxoestradiol, and 15α-hydroxyestrone but caused an increase in the fecal excretion of both conjugated and unconjugated estriol. These workers demonstrated a significant change in the pattern and level of the various estrogens after treatment with ampicillin. They also showed that treatment with ampicillin reduced the level of urinary pregnanediol glucosiduronate and increased the fecal conjugates of progesterone.[134]

Erikssen et al.[290-292] studied the effect of intestinal microflora on the metabolism of C_{19} and C_{21} steroids in rats and found that the metabolism is significantly different in germ-free rats compared with conventional rats. These differences were explained as being due to the direct participation of the intestinal microflora. Amland and Stoa[293] have investigated the role of the intestinal bacteria on the metabolism of the phenolic C_{18} steroids in the rat and found up to 42% of the 17β-estradiol converted to estrone by an intestinal culture from adult male rats.

The effect of diet on the biosynthesis, metabolism, and excretion of steroid conjugates is also thought to be mediated through alterations in the intestinal microflora.[274,275] The qualitative and quantitative differences in the kind of bacteria present in the intestinal tract seem to be controlled, at least in part, by the dietary intake.[276-278] The influence of diet on the incidence of breast[294] and colon cancer[295] has been documented from epidemiological studies. This led some workers to postulate a relationship between diet and breast and colon cancer that is mediated through the effect of the intestinal microflora on the metabolism of steroids in the gut.

A number of other factors may also control or alter in vivo levels of β-glucuronidase, uridine diphosphoglucuronic acid, and steroid glucuronyl transferases, thereby modifying steroid conjugation, metabolism, and excretion. These factors have been discussed in a previous review.[296]

VIII. TRANSPORT AND EXCRETION OF STEROID CONJUGATES

A. Plasma Protein Binding

Steroid hormones are transported primarily via the blood both in the unconjugated and conjugated form. Since unconjugated steroids have rather low aqueous solubility, binding to plasma proteins was assumed to be necessary to facilitate transport in plasma. This conclusion was drawn from early studies where large doses of steroids were used. More recent studies indicate that the physiological concentrations of steroids in plasma are well below the limits of solubility.[1,296] Furthermore, the occurrence of conjugated steroids in plasma[297] and the high solubility of steroid conjugates in aqueous solutions suggest that transport may not be the primary function of steroid binding proteins. The protein-steroid complex may function to protect the steroid from catabolism and to act as a "buffer system" for steroid levels in the extracellular space.[298]

Various steroids and steroid conjugates are known to be associated with plasma proteins.[298-302] The plasma binding of unconjugated steroids has been recently reviewed.[299] Despite the relatively high solubility of steroid conjugates in aqueous solutions, there is considerable evidence that they may also be bound to plasma proteins.[298,303-305] Numerous studies have established that the major types of estrogen conjugates present in both plasma and urine are sulfates or glucosiduronates. However, the ratio of conjugates in these two fluids is very different. The ratio of estrogen sulfates/glucosiduronates is greater than 4 in plasma but less than 1 in the urine.[63,202] Recent work with endogenous levels of individual estrogen sulfates and glucosiduronates confirms this pattern of higher sulfate levels in plasma and higher glucosiduronate levels in urine.[174-176] These differences in plasma and urinary levels of sulfates and glucosiduronates are attributed in part to differences in binding to plasma proteins.[202]

Slaunwhite and Sandberg[1,298] showed that estrogen glucosiduronates bind primarily to albumin in human plasma. The differential binding of sulfates and glucosiduronates was demonstrated by Rosenthal et al.[303,304] and Goebelsmann et al.[305] Estrogen sulfates are more strongly bound to albumin than estrogen glucosiduronates. They concluded that the differences in metabolic clearance rates could be explained by the lower binding by albumin of the glucosiduronates.

B. Transport Across the Feto-Placental Membrane

The amount of steroids and steroid conjugates excreted in human maternal urine increases during pregnancy and is largely attributable to the metabolic activity of the fetus and placenta, especially during the third trimester. The fetus is rich in sulfokinases which are capable of sulfating a variety of steroids.[245] In contrast, the placenta is largely devoid of sulfokinases but is rich in sulfatases.[245] Following placental perfusion with cholesterol-3β-sulfate, 50 to 70% of the radioactivity was ether extractable, indicating the presence of sulfatase activity in the placenta.[306] Significant amounts of free pregnenolone and progesterone were found following *in situ* perfusion of full or midterm placentae with pregnenolone sulfate.[306-308] Considerable specificity for placental sulfatase enzymes was noted. Sulfates at the 3β-hydroxy position with a Δ^5 function[224,225,309-313] and phenolic sulfates of estrogens[83,314-316] are good substrates for placental sulfatase activity, whereas steroid sulfate esters such as 16α-hydroxyprogesterone-16-sulfate, testosterone sulfate, and androsterone-3α-sulfate and corticosterone-21-sulfate[312,313] are relatively poor substrates.

Unlike the sulfates, steroid glucosiduronates are fairly stable within the feto-placental unit. Furthermore, the estrogen glucosiduronates can be metabolized without removal of the glucosiduronate group. When Zucconi et al.[194] perfused the feto-placental unit with radioactive estrone glucosiduronate, 17β-estradiol glucosiduronates and estriol glucosiduronates were obtained without any evidence of prior hydrolysis.

Goebelsmann et al.[317] administered ^{3}H-estriol sulfate and ^{14}C-estriol-16α-glucosiduronate into the amniotic cavity of a pregnant woman and found more than 50% of the tritium but only 16% of the ^{14}C in the maternal urine within 96 hr. These results suggest that estriol sulfate is transferred across the placental membrane more rapidly than the glucosiduronates. Other studies confirmed that estriol-16α-glucosiduronate[318] and estriol-3-glucosiduronate[319] cross the placental membranes without hydrolysis, indicating low β-glucuronidase activity in the placenta. A comprehensive review of the biosynthesis and metabolism of steroids in the feto-placental unit has appeared.[245]

The transfer of steroids between the maternal and fetal compartments across the placental barrier depends upon whether the steroids are conjugated and the type of conjugates. Unconjugated steroids cross the barrier more freely than steroid conjugates. Superimposed upon this limitation are the intense sulfatase activity of the placenta and the ubiquitous sulfokinases present in the fetal tissues. Thus, steroids which are transferred or cross the placenta to the fetus are mostly in the unconjugated form, while those transferred from the fetus are present as steroid sulfates. Steroid glucosiduronates cross the placental barrier without hydrolysis, but at a much slower rate than the steroid sulfates. The effects of these factors on the transfer of estriol and estriol conjugates through the amniotic and chorionic membranes of the human fetus were reported by Katz et al.,[320] who observed that unconjugated estriol easily crossed the membranes but estriol-16α-glucosiduronate and estriol-3-sulfate did not. Moreover, the transfer of free estriol was more rapid through the amniotic than through the chorionic membranes. The authors suggested these differences in the transfer of free and conjugated estrogens were a function of the physiochemical properties of the membranes, which include impermeability to water soluble substances, such as steroid conjugates,[321] and the electronegative character of the steroid conjugates.[322] Other investigators, Levitz et al.[323] and Dancis et al.,[324] confirmed these results with guinea pig placenta. Unconjugated estrogen readily crossed the guinea pig placenta, but it was impermeable to estrone sulfate.

IX. STEROID DYNAMICS

The study of steroid dynamics refers principally to the rates of steroid metabolism in its various assests and by extension to the rates of steroid production There are

two principal techniques available for measuring these parameters, namely those involving measurements in the blood and those involving measurements in the urine. In the "blood" method, either a single intravenous injection (S) or a constant rate intravenous infusion (I) of a known amount of radiolabeled steroid is given. All of the following parameters may be measured by the single injection technique, and some can also be measured by the constant infusion technique.

1. Initial volume of distribution (S)
2. Half life (S)
3. Disappearance rate (S)
4. Metabolic clearance rate — MCR (S or I)
5. Rate constant for total removal (S)
6. Proportion of steroid irreversibly cleared (S)
7. Proportion reversibly cleared (S)
8. Conversion ratio (S or I)
9. With simultaneously measured endogenous plasma level — production rate — PR (S or I)
10. With simultaneous endogenous plasma levels of a precursor and a product — Δ = proportion of a product formed from a precursor (S or I)
11. With simultaneous measured MCR of a precursor or a product — ϱ value = proportion of a precursor converted to a product (S or I)

A further and related technique is useful in studying the uptake, release, and interconversion rates of steroids by individual tissues or areas. Simple uptake and/or release by tissues can be calculated from arterial (A) - venous (V) plasma concentration differences, either of endogenous steroids or of radiolabeled steroids after the appropriate infusions. The percent extraction [100 (1 − V/A)] can also be calculated. It should be noted that uptake by an area can be due to metabolism, excretion, or removal from the site by nonexcretory routes other than the efferent vein (e.g., the lymphatics). Release indicates net formation in the area for release into the efferent vein. It should also be noted that arterio-venous differences and extractions estimated in this way are *net* effects, since several of these processes may be going on simultaneously. These aspects have been previously discussed (see regional metabolism and hydrolysis).

Study of the dynamics of estrogens, androgens and glucocorticoids, has yielded much valuable information regarding the secretion, metabolism and interconversions of these hormones. However, the studies have been mostly confined to nonconjugated steroids, with the notable exception of dehydroisoandrosterone sulfate, which has been studied by "urinary" methods. In the estrogen field there have been a few published studies of estrogen conjugate dynamics, namely, those of estrone sulfate in the human[54,55] and of estrone glucosiduronate in the dog.[56] The dynamics of testosterone sulfate in the human have also been reported.[325,326]

The results of measurements of estrone sulfate dynamics in the human are given in Tables 10 and 11. In Table 10, values for metabolic clearance rates (MCR) and production rates (PR) are given, together with corresponding results for the principal unconjugated estrogens, estrone,[327,328] 17β-estradiol,[327,328] and estriol,[329] for comparison. The MCR for estrone sulfate in males and females are similar, and both are one tenth or less than the MCR for estrone, 17β-estradiol, and estriol. The ϱ values for estrone sulfate were determined by two different groups (Table 11).[54,55] The ϱ value for $E_1S \rightarrow E_1$ is about 0.18, but the ϱ value for $E_1S \rightarrow E_2$ is much lower (0.018). The ϱ values $E_1 \rightarrow E_1S$, and $E_2 \rightarrow E_1S$ are similar, both being about 0.50. These results led both laboratories to conclude that the plasma levels of estrone sulfate can be accounted for on the basis of the above mentioned conversions and that it is not necessary to postulate secretion of estrone sulfate by the gonad or adrenal.

TABLE 10

Mean Metabolic Clearance Rate ± SE (MCR) and Mean Production Rate ± SE (PR) of Various Unconjugated Estrogens and of Estrone Sulfate

	MCR (1/day/m²)		PR (μg/day)		
Compound	Females	Males	Females	Males	Ref.
Estrone	1120 ± 80[a]		101 ± 13[a]		327
	1320 ± 70[b]	1310 ± 80			328
17β-Estradiol	740 ± 50[a]		80 ± 11[a]		327
	790 ± 20[b]	990 ± 50			328
Estriol	1240 ± 40[a]		14 ± 1.6[a]		329
	1280[a] (65)[c]		23 ± 1.9[c]		
Estrone sulfate	94 ± 22[d]	87 ± 21	95[e] 182[e]	77	54
		90 ± 10			55

[a] Follicular phase.
[b] Grouped follicular and luteal phase.
[c] Luteal phase.
[d] Time of menstrual cycle not given.
[e] Estimated.

TABLE 11

ϱ Values of Estrone Sulfate

	Males and females[a,54]	Males[55]
$E_1S \rightarrow E_1$	0.21 (0. 13—0.30)[a]	0.15 (0.12—0.18)[b]
$E_1S \rightarrow E_2$	0.014 (0.012—0.10)[a]	0.022, 0.44[c]
$E_1 \rightarrow E_1S$	0.54 (0.38—0.67)[a]	0.41 (0.21—0.81)[b]
$E_2 \rightarrow E_1S$	0.65 (0.22—1.20)[a]	0.42 (0.30—0.58)[b]

[a] Range in parentheses.
[b] 95% confidence limits in parentheses.
[c] Two subjects only.

The regional metabolism and interconversions of estrone sulfate in the human forearm have been studied by Longcope et al.[261] The observed ϱ values (fraction of estrone sulfate in arterial blood metabolized by muscle [as opposed to adipose tissue]) were 0.04 and 0.03 in two subjects. Conversion of estrone sulfate to estrone was shown to occur in the forearm in only one of three subjects, and in no case was there conversion of estrone sulfate to 17β-estradiol. Conversion of estrone to estrone sulfate by the forearm was only observed in one case, and in no instance was there conversion of 17β-estradiol to estrone sulfate. Thus, the forearm seems to be of minor importance in these conversions of estrone sulfate.

The dynamics of the other principal conjugated metabolite of estrone, namely estrone glucosiduronate, have not been studied in the human but have been studied in the dog by Collins et al.[56] The MCR ± SE of estrone glucosiduronate in a series of dogs was 329 ± 35 ℓ/day/m², substantially less than the MCR for estrone in the same animal (731 ± 50ℓ/day/m²).[255] The observed mean ϱ value ± SE for conversion of estrone glucosiduronate → estrone in the dog was 0.057 ± 0.006, which is somewhat lower than that for estrone sulfate → estrone in the human (Table 11). The calculated

ϱ value for estrone → estrone glucosiduronate was 0.6, which was very similar to that for estrone → estrone sulfate in the human.

The dynamics of testosterone sulfate have been studied by Wang et al.[325] and Saez et al.,[326] and the results are shown in Table 12. The whole blood MCR was determined in two men and compared with the MCR for testosterone in the same subjects by Wang et al.[325] Saez et al.[326] measured the plasma MCR in four normal males and also estimated the "blood" PR. Both groups observed that the MCR of testosterone sulfate is substantially less than that for testosterone, although there is some discrepancy between the two sets of values obtained.

The dynamics of dehydroisoandrosterone sulfate have been compared with those of dehydroisoandrosterone in the human male by Wang et al.[325] and by Sandberg et al.[330] (Table 12). Again, the MCR for the sulfate is substantially less than for the parent compound, dehydroisoandrosterone. Thus we have evidence in the human that the MCRs of the sulfates of estrone, testosterone, and dehydroisoandrosterone are all substantially less than those of the corresponding free steroid. In addition, the MCR of estrone glucosiduronate in the dog is also considerably less than the MCR of estrone.[56] Whether this is part of a general pattern and whether this is in turn dependent on differences in plasma protein binding, is not known.

In considering the MCRs of estrogen conjugates (and indeed other steroid conjugates) the role of renal excretion has to be borne in mind. Renal excretion of unconjugated estrogens is small and can be ignored, but this is not necessarily the case for estrogen conjugates where renal excretion may be substantial. This is especially true with the glucosiduronates where active renal secretion may be involved. Since the metabolic clearance rate is defined as the rate or irreversible disappearance of a substance from the blood, a substantial proportion of this might be due to disappearance via renal excretion, rather than any metabolic process.

"Urinary" methods were used by MacDonald and co-workers[331] in their analysis of the dynamics of dehydroisoandrosterone sulfate. Urinary methods generally consist of the administration of a radiolabeled steroid as a single intravenous injection and collection of the urine until all recoverable radioactivity has been excreted. As an extension of this technique, two related steroids with different radiolabels (e.g., ^{3}H, ^{14}C) can be injected and interconversions estimated. The technique yields secretion and production rate estimates, ϱ values, and various rates of metabolism. Urinary methods are, in general, more convenient than the blood methods, but some of the parameters involve assumptions regarding the origin of the radioactive metabolites measured, while others are dependent on the assumption of a certain model. However, some parameters (e.g., ϱ values) can be measured independently of the model. The results of the studies of MacDonald et al.[331] are given in Table 12.

X. SUMMARY

There is now considerable evidence that the conjugation of steroids occurs in a number of different organs and has important effects on their transport, excretion and tissue distribution.

Liver, kidney, small intestine, lung, breast, and the feto-placental unit have been shown to have the enzymes necessary for the formation of steroid conjugates. Muscle, some endocrine organs such as the adrenal, ovary and testis, and the endometrium have also been reported to form steroid conjugates under in vitro conditions. The liver is by far the most active organ in the conjugation of steroids and is capable of forming glucosiduronates of all steroids thus far studied. In addition, several species can form other conjugates such as sulfates, N-acetylglucosaminides, glucosides, and glutathiones as well as double conjugates.

TABLE 12

Metabolic Clearance Rates (MCR), Secretion Rates (SR), and Production Rates (PR) for Testosterone Sulfate (TS), Testosterone (T), Dehydroisoandrosterone Sulfate (DHAS), and Dehydroisoandrosterone (DHA), Together with ϱ Values for DHAS → DHA and DHA → DHAS in the Human

	n[a]	TS	T	Ref.
MCR (l/day/m²) (whole blood, males)	2	21.5—24.5	775—2029	325
MCR (l/day/m²) (plasma, males)	4	75—240	560—1180	326 332
"Blood" PR (μg/day) (males)	2	64—310	6480—801.5	326 332
		DHAS	DHA	
Plasma MCR (l/day/m²)	1	7.7		330
Whole blood MCR (l/day/m²)	3	16.5—32.7		325
	2		854—1059	325
"Urinary" SR (mg/day)	4	7.7—23.4	0.7—9.1	331
"Urinary" PR (mg/day)	4	8.1—27.8	6.9—17.2	331
ϱ DHAS → DHA	1	0.63		330
	4	0.31—0.81		331
ϱ DHA → DHAS	1	0.48		330
	4	0.44—0.59		331

[a] Number of subjects.

The major metabolic product after incubation of estrone and 17β-estradiol with liver homogenates from nonhuman primates such as the baboon, chimpanzee and rhesus monkey is 17β-estradiol-17-glucosiduronate. However, the ability of the human liver to form 17β-estradiol-17-glucosiduronate is still controversial.

The sulfokinases of liver are particularily active in the conjugation of phenolic and neutral steroids in all species studied. The liver of the rat and hen are unique in their ability to form disulfates of estradiol. Sulfate formation may be a mechanism for storage of the steroids in an inactive form. The presence of sulfatases in a number of tissues capable of hydrolyzing estrone sulfate suggests that sulfates may be a source of physiologically active estrogens. There is good evidence that estrone sulfate may be the primary metabolite of 17β-estradiol and the principal intermediate to other metabolites.

The transfer of N-acetylglucosamine and glucose to phenolic and alcoholic groups is accomplished by a number of different species such as the rabbit, sheep, chimpanzee, as well as the human. These species show significant qualitative and quantitative differences in their ability to form N-acetylglucosamine or glucose conjugates. In the sheep and rabbit, conjugates containing N-acetylglucosamine predominate, whereas the ability of the chimpanzee liver in vitro to transfer glucose to a steroid hydroxyl group exceeds its ability to transfer glucosiduronic acid.

Other tissues have also been reported to be active in the formation of steroid conjugates. Although the activity is lower in the kidney than the liver, the pattern of conjugation is similar for estrogens. One different route of metabolism is the conjugation

of aldosterone with an acid labile conjugate which may be directly excreted by the kidney.

The intestinal mucosa has been shown to be an important site for conjugation of estrogens in both the rat and human. The fetal and neonatal lungs convert estrogens, adrenocorticoids and progestins to their corresponding sulfates. However, the adult lung does not seem to have significant sulfotransferase activity. The formation of dehydroisoandrosterone sulfate and other sulfates has been demonstrated using adrenal tissue preparations.

One of the most significant advances in the study of steroid conjugates in recent years has been the development of sensitive and direct methods for measuring specific steroid conjugates in biological fluids. Specific antisera have been reported for testosterone glucosiduronate, dehydroisoandrosterone sulfate, estriol-16α-glucosiduronate, estrone glucosiduronate, 17β-estradiol-17-glucosiduronate and estrone sulfate which allow direct analysis by radioimmunoassay. In addition, a number of methods involving chromatographic separation of the specific steroid conjugates followed by hydrolysis and radioimmunoassay of the free estrogen have been reported. These new methods have greatly increased our understanding of the endogenous levels of steroid conjugates in various biological fluids.

The conjugation of testosterone may be summarized as follows: The unconjugated steroid is the principal plasma component in men; the plasma concentration of the glucosiduronate is higher than the sulfate. The sulfate, but not the glucosiduronate, is secreted by the testis. The plasma concentration of the unconjugated testosterone and testosterone glucosiduronate are similar in women and both are greater than the sulfate. The urinary excretion of the glucosiduronate of testosterone is much greater than the sulfate in both men and women.

Dehydroisoandrosterone sulfate is secreted primarily by the adrenal and does not show any variation with sex or reproductive status. There is variation with age, the highest levels being reached between 20 to 30 yr followed by a steady decline. Dehydroisoandrosterone sulfate is the principal urinary conjugate being about five times as much as dehydroisoandrosterone glucosiduronate.

Estriol-16α-glucosiduronate is the major urinary estrogen in pregnancy. Considerable interest has centered on its potential for monitoring pregnancies. The values reported from different laboratories are similar, increasing from less than 1 mg/24 hr in early pregnancy to 40 to 50 mg/24 hr at term. Plasma levels of estriol-16α-glucosiduronate increase throughout pregnancy and exhibit a marked rise from 31 to 41 weeks of pregnancy. The plasma levels are 2 to 3 times those reported for estriol, and suggest that plasma estriol-16α-glucosiduronate can effectively be used to measure fetal well-being.

The urinary and plasma levels of estrone glucosiduronate, 17β-estradiol-17-glucosiduronate, estriol-16α-glucosiduronate, and estrone sulfate in normal men and women have been reported. Estrone sulfate is the major estrogen conjugate present in plasma whereas the other estrogen conjugates are very low.

Considerable evidence has accumulated to indicate that steroid conjugates can be extensively metabolized before excretion. Furthermore, the steroid moiety may undergo further metabolism while the glycosidic or ester linkage is often preserved.

A large number of steroids are conjugated by the liver and subsequently excreted into the small intestine via the bile. These steroid conjugates are hydrolyzed by the bacteria in the gut, absorbed and reconjugated by the intestinal wall and transported to the liver via the portal vein. This closed circuit is interrupted by the hepatocytes which store certain steroid conjugates for subsequent release into the blood where they are quickly excreted by the kidney. Double conjugates of the estrogens are preferrentially excreted into the bile while glucosiduronates are primarily released into the blood and excreted primarily in the urine.

The liver converts estriol to the monoconjugates, estriol-3-sulfate and estriol-16α-glucosiduronate and the double conjugate estriol-3-sulfate, 16α-glucosiduronate which are excreted in both the urine and bile. The conjugates excreted in the bile undergo hydrolysis in the gut to yield free estriol which is reabsorbed into the intestinal mucosa and reconjugated with glucosiduronic acid at the 3- and 16α-position. The estriol-3-glucosiduronate is quantitatively excreted into the urine while estriol-16α-glucosiduronate is in part converted to the double conjugate, estriol-3-sulfate, 16α-glucosiduronate in the liver and excreted again into the bile.

One of the major metabolic steps for steroid conjugates is hydrolysis of the conjugating group to give the free steroid. A major site of hydrolysis is the duodenal region of the intestinal tract as mentioned above. In vivo hydrolysis of estrone sulfate as well as of estrone and 17β-estradiol glucosiduronates have been demonstrated. Additional studies suggest that the lungs are active in the hydrolysis of the estrogen glucosiduronates.

The formation and metabolism of steroid conjugates by various organs has been determined. The unconjugated estrogens, estrone, 17β-estradiol and estriol are taken up by the splanchnic area, intestine, spleen (estrone only studied), and hind limb. In contrast, estrone and 17β-estradiol (but not estriol) are formed by hydrolysis of estrogen glucosiduronates in the lungs for release into the pulmonary vein. The estrogen conjugates, estrone glucosiduronate, 17β-estradiol glucosiduronate(s), estriol-16α-glucosiduronate and estriol-3-sulfate, 16α-glucosiduronate are formed in the liver and intestine. There is net uptake of estrone glucosiduronate by the spleen, lungs and hind limb and of 17β-estradiol glucosiduronate(s) by the lungs, presumably reflecting metabolism including hydrolysis. The formation and metabolism of estrone sulfate appears to be somewhat different from other estrogen conjugates, but has not yet been fully investigated.

The formation and metabolism of steroid conjugates is altered by disease states, drugs and diet. Total biliary obstruction leads to urinary excretion of steroid conjugates that normally enter the intestine via the bile. Antibiotics are known to alter the intestinal flora drastically, resulting in reduction in the enteric hydrolysis and reconjugation of biliary steroid conjugates. The effects of diet on the biosynthesis, metabolism and excretion of steroid conjugates is also thought to be mediated through alterations in the intestinal microflora.

Study of steroid dynamics yields important information about the secretion, metabolism, excretion, or interconversion of dehydroisoandrosterone sulfate, estrone sulfate, estrone glucosiduronate, and testosterone sulfate. The metabolic clearances for all of these conjugates were substantially less than the free steroid.

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Chapter 4

HYDROXYLATED C_{18} AND C_{19} STEROIDS; THEIR SIGNIFICANCE AND FACTORS RELATED TO THEIR BIOSYNTHESIS

R. Hobkirk

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I. INTRODUCTION

Biological hydroxylation of steroids has been recognized for many years. Hydroxylation at a variety of carbon atoms in the steroid molecule plays a well-defined role in the biosynthesis of such important hormones as the adrenal corticosteroids. A much more poorly understood role for hydroxylation is evident during metabolism of steroids containing 18 and 19 carbon atoms, i.e., the "androgen" and "estrogen" series. Earlier views favored hydroxylation of these latter steroids as part of an overall catabolic or inactivating mechanism resulting in removal of the active hormone from the biological scene. Some recent evidence, however, may conceivably indicate that certain hydroxylations can lead to the formation of steroids which are not totally lacking in neutral steroids. It might be argued teleologically that the variety of carbon atoms subjected to hydroxylation in the estrogen and androgen series must have some biological significance besides direct inactivation. If not, surely simple detoxification could be achieved by a single hydroxylation followed, perhaps, by formation of a glucuronide conjugate for purposes of rapid excretion. The situation with regard to the C_{18} and C_{19} steroids has been further complicated by the demonstration that prior conjugation of these in the sulfate form can markedly affect, in both a qualitative and quantitative manner, subsequent hydroxylation(s).

This chapter will discuss some aspects of the biological hydroxylation of the estrogens and androgens, including a review of the relationship of various hydroxylation steps, to the biological importance of the steroid metabolites produced. In addition, the effect of steroid substrate conjugation upon the activities of the hydroxylases will be considered. In so doing, some recent data from the author's laboratory will be presented along with available information published by other investigators. The material considered will be mainly concerned with mammalian species.

II. C_{18} STEROIDS (ESTROGENS)

A. Hydroxylated Forms and their Biological Importance

The C_{18} phenolic steroids may be hydroxylated at several molecular positions. The number and type of metabolites formed varies from one mammalian species to another. Thus (to mention only a few examples), human,[1-3] rat,[4,5] and mouse[6] are capable of forming a considerable number of hydroxylated metabolites whereas hamster,[7] rabbit,[8,9] and guinea pig[10] produce only a few, or none at all, depending upon the experimental approach employed in the study. When one takes into account both in vivo and in vitro experimentation in all species investigated, it becomes evident that hydroxylation may occur at positions 2, 4, 6, 7, 11, 14, 15, 16, and 18. Moreover, either α- or β-isomers, or both, may arise through hydroxylation at carbons 6, 7, 15, and 16 of the steroid molecule.

Up to the present, little information is available to explain the raison d' etre of these many steroidal metabolites, in a biological sense. Although the 16α-hydroxylated phenolic steroid, estriol [estra- 1,3,5(10)-triene-3, 16α, 17β-triol], was first isolated in 1930,[11] its function is still being sought, particularly in the human during pregnancy when vast amounts of it are produced.[12,13] Over the years, a number of investigators have drawn attention to the biological activity of this steroid (for an earlier review, see Reference 14). Recent information indicates that estriol (usually considered to have weak estrogenic action) may, under certain circumstances, function as an active hormone in the uterus. This is related to the much shorter uterine half-life of estriol compared with that of the highly potent estradiol-17β[15,16] [estra-1,3,5(10)-triene-3,17β-diol]. The affinity of estriol for the estrogen receptor is also considerably lower than that of estradiol.[17] Thus, in a situation where uterine tissue is exposed to a short pulse of steroid, estriol exhibits a lesser uterotropic effect than does estradiol. This difference

has been reported to result not from failure to stimulate critical stages of growth, but rather, from a failure to sustain the products of growth.[18] Indeed, under these circumstances, estriol may behave as an antiestrogen.[19-21] In the presence of a continuous production of estriol, estrogenic activity prevails due to residency on the uterine receptor despite the short nuclear retention time.[18,21,22] Uterine chromatin appears to possess about as many binding sites for estriol as it does for estradiol.[17] It has been demonstrated[23] that during superfusion of human uterine endometrium an estriol/estradiol intracellular ratio of approximately 2 results in a 50% displacement of the latter hormone from the nuclear receptor. The relative uterotropic activities of estradiol and estriol are influenced by the presence of extracellular proteins capable of steroid binding, a factor frequently overlooked in in vitro experimentation. Thus, Anderson et al.[24] have shown that, at a concentration of 2 n*M* in the absence of serum albumin, estradiol half saturates the nuclear receptor of immature rat uterus. A similar degree of saturation is observed for estriol at a 6 n*M* concentration. In the presence of serum albumin, 18 n*M* estradiol and 9 n*M* estriol are required, respectively, to achieve a 50% receptor saturation. Obviously, such phenomena must occur in the in vivo situation.

This information allows one to consider estriol as a potentially active estrogen under certain circumstances. One might also note the reported influence of estriol in protecting against certain carcinogen-induced mammary tumors.[25] Consequently, the 16α-hydroxylation leading to estriol formation, particularly in the human where it is a major pathway, may be of considerable importance. A better understanding of the mechanism involved and the factors which may regulate it should be most useful.

Hydroxylation of C_{18} phenolic steroids at position 2 has attracted considerable attention since the demonstration that 2-hydroxyestrogens (catechol estrogens) are major metabolites in several species,[5-7,26] including the human.[2,27,28] These compounds possess certain biological activities unrelated to their uterotropic functions. Thus, Gordon et al.[29] have shown that 2-hydroxyestrone [2,3-dihydroxyestra-1,3,5(10)-trien-17-one] and 2-hydroxyestriol [estra-1,3,5(10)-triene-2,3,16α,17β-tetrol] possess 28 and 59%, respectively, of the hypocholesterolemic activity of estradiol when administered orally to the male rat. These same two steroids exhibit only 0.14% of the uterotropic activity of estradiol in the immature female rat. Even more striking is the case of 2-methoxyestriol [2-methyl-estra-1,3,5(10)-triene-2,3,16α,17β-tetrol], which was reported to possess 207% of the hypocholesterolemic activity of estradiol but only 0.05% of the latter's uterotropic activity. It should also be mentioned that a 16-oxygenated metabolite, 16-ketoestradiol-17β[3,17β-dihydroxyestra-1,3,5(10)-trien-16-one] — while exhibiting 138% of the hypocholesterolemic activity of estradiol — appears to be considerably more uterotropic (2.2% of estradiol activity) than any of the 2-oxygenated steroids.[29]

The catechol estrogens structurally resemble epinephrine and norepinephrine in that they possess the o-quinol structure. These steroids are capable of competing with the catecholamines[30,31] for the enzyme catechol-O-methyl transferase (EC 2.1.1.a) which plays an important role in the inactivation of the amines in question. This has led investigators to propose that the 2-hydroxy phenolic steroids, which lead to an increased half-life of the active amines, could be involved in the etiology of certain hypertensive disorders.[32,33] A further relationship between this interesting class of steroids and the central nervous system (CNS) has arisen from the studies reported by Naftolin et al.[34] in which subcutaneously administered 2-hydroxyestrone, but not estrone, caused a dramatic rise in serum LH in the immature male rat. In a separate study,[35] it was found that either 2-hydroxyestrone or estrone could considerably stimulate both plasma LH and FSH in oophorectomized adult rats whose gonadotropin levels had been suppressed by priming with estradiol. It has also been claimed[36] that 2-hydroxyestradiol [estra-1,3,5(10)-triene-2,3,17β-triol] led to a fall in plasma LH when introduced into the amygdala of orchidectomized minipigs. These data, as well as other findings of 2-hydroxylation of estrogens by CNS tissues from rat[37] and human fetuses[38] together

with the presence of high-affinity binding sites for 2-hydroxyestrone and 2-hydroxyestradiol in high-speed supernatants of rat pituitary and anterior hypothalamus,[39] point to important functions for the catechol estrogens.

The binding affinity of rat uterine cytosol and chromatin preparations for 2-hydroxyestradiol is reportedly 25% that of estradiol.[40] This binding does not appear to be related to an estrogenic response, since the catechol estrogen exhibits < 1% of the uterotropic activity of estradiol.[41] Moreover, 2-hydroxyestrone, with only 0.1% of the uterotropic activity of estrone,[42] has been shown[40] to possess 20% of the binding affinity of the latter steroid. In contrast to this, certain other steroids showing little or no uterotropic activity, such as 2-methoxy phenolic steroids and estetrol [estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol], are bound scarcely at all to the rat uterine receptors in vitro. It has been suggested[40] that these data imply an antiestrogenic role for the 2-hydroxysteroids. It is of interest to note that, in a study reported in 1955,[43] 2-hydroxyestradiol was demonstrated to possess 1% of the uterotropic activity of estradiol in vivo in the oophorectomized rat and in the mouse. Nevertheless, the same catechol estrogen considerably stimulated formate incorporation into uterine protein in vitro as compared with the effect of estradiol.

Basic biochemical studies have shown[44] that 2-hydroxyestradiol can interfere in NADPH-dependent electron transport of rat liver microsomes by inhibiting cytochrome P450 reduction, cytochrome b_5 reduction, and lipid peroxidation. Both 2-hydroxyestrone and 2-hydroxyestradiol are potent inhibitors of rat liver microsomal conversion of estrone to water-soluble metabolites.[45] Estradiol-17β, estradiol-17α, and 17α-ethynylestradiol have a lesser effect, whereas steroids hydroxylated at positions 6 or 16 are inactive.

Thus, there is compelling evidence to suggest that the catechol estrogens have biological roles and that these functions may be quite distinct from those of the 16-hydroxy phenolic steroids, on the one hand, and the parent estrogen, estradiol, on the other.

In considering the activities of the various C_{18} phenolic steroids, it should not be overlooked that all estrogen-sensitive tissues are not necessarily affected to the same degree by a given steroid. Moreover, the relative effects may differ between animal species. 16-Ketoestradiol seems to be lacking major activity in the uterus,[46] but repeated administration of this steroid is stimulatory towards mouse vaginal tissue.[47] Through the use of the mouse intravaginal tetrazolium blue bioassay for estrogens,[48] involving measurement of the effect of applied steroid upon the levels of tissue enzymes, Martin[49] noted the considerable activity of 17-epiestriol [estra-1,3,5(10)-triene-3,16α,17α-triol]. The dose-response curve for this compound has a similar slope to that of estradiol and estrone and, in terms of absolute activity, lies between those of the two latter steroids. This is in marked contrast to other 16-oxygenated steroids such as estriol and 16-epiestriol [estra-1,3,5(10)-triene-3,16β,17β-triol], which exhibit low biological activity with shallow dose-response curves typical of the "impeded" estrogens. To date, no study seems to have been reported as to the uterotropic activity of 17-epiestriol. Using the latter bioassay, one finds that the 2-hydroxy and 2-methoxy phenolic steroids exhibit extremely low activity but possess curve slopes similar to those of estradiol and estrone.

With the exception of the 2- and 16-hydroxylated phenolic steroids, there is little information as to the biological function of the many hydroxylated forms known to be naturally occurring. The relationship of hydroxylation at any specific position of the steroid molecule to a particular biological function is unclear. Relatively recent evidence[50] for the formation of 4-hydroxylated estrogens in the human (albeit in small amount), coupled with the information[40] that 4-hydroxyestradiol [estra-1,3,5(10)-triene-3,4,17β-triol] is bound by rat uterine cytosol receptor to the extent of 43% that of estradiol (cf., 23% for 2-hydroxyestradiol), suggests an avenue for exploration. It

has also been reported[43] that 4-hydroxyestradiol stimulates formate incorporation into protein of rat and mouse uterine tissue in vitro to a much greater degree than does estradiol, although the latter hormone has ten times more uterotropic activity. Rat liver preparations are also known[51] to form pyrogallolestrogens (i.e., 2,3,4-trihydroxy compounds) from the catechol estrogens. These, as well as certain of their methyl ethers, can inhibit the catechol-O-methyl transferase of rat liver.[52] Thus, 4-hydroxylation may prove to be of some importance.

Considerable interest has centered around the formation of 15-hydroxysteroids, largely due to research indicating[53,54] that the urinary metabolite estetrol has its origin mainly in the fetal rather than maternal compartment in human pregnancy. Attempts have thus been made[55,56] to employ the measurement of estetrol as an index of fetal viability. Overall, this does not appear to have offered any advantage over the measurement of estriol, which is quantitatively the main estrogen metabolite of the human fetoplacental unit.[57] With respect to the 15α-hydroxylated forms of the phenolic steroids, it is noteworthy that in superfusion experiments with human uterine endometrium[58] estetrol can displace 65% of intranuclear estradiol. This may appear to be at variance with the in vitro binding studies mentioned above.[40] However, it has been pointed out[58] that the ability of steroids to compete for nuclear binding sites is very dependent upon their concentrations achieved within the cell in whole tissue or whole cell preparations. Consequently, the 15-hydroxylated steroids may be found to possess some biological activity. Thus far, however, there seems to be no firm basis for ascribing any particular function, except perhaps detoxification, to phenolic steroids possessing oxygen functions at positions 6, 7, 11, 14, or 18.

Upon consideration of the great qualitative variability in hydroxylated metabolites formed by various species, one may speculate that if certain of these steroids are required in a given species they are not indispensable in others. However, such a view must be carefully examined in the light of the experimental approach employed in each study. It is not unusual, for example, to identify a number of steroid metabolites under in vitro conditions using certain animal tissues or fractions thereof, while failing to detect formation of these same compounds by the corresponding species in vivo. This could result from failure to consider all the possible excretory routes of, or all of the possible tissues containing, the metabolites in the in vivo situation. Additionally, in acute in vivo experiments, the dynamics of the system do not always allow time for production of detectable amounts of the metabolite sought prior to wide and rapid tissue distribution and excretion. Alternatively, in the in vitro approach, whether working with slices, minces, homogenates, or subcellular fractions, one may be exposing substrates to enzymes in a fashion which is never approached in vivo.

Even with the above qualifications in mind, however, there is considerable variation in the metabolites formed from one species to another and, of course, from one tissue to another. In the nonpregnant human, 2-hydroxylation is reported[2,27,28] to be the main pathway, while in pregnancy, 16α-hydroxylation by fetal liver is the major metabolic route,[57] although much of the latter represents hydroxylation of neutral steroids followed by aromatization. In addition, many human tissues hydroxylate at various positions of the steroid molecule, including 6α-hydroxylation by the placenta[59] and 15α- and 18-hydroxylation by the adrenal.[60] The human fetal adrenal has been reported[61] to hydroxylate at 2, 7α, 15α, and 16α. The major estrogen metabolite identified[26,62] in rat urine and bile is 2-hydroxyestrone, while, from in vitro experiments using whole liver, 2-, 6-, and 16-hydroxylation, (in particular) have been detected.[4,5,63] In addition, rat liver microsomes may form many hydroxylated metabolites.[64] Mouse liver slices hydroxylate at 2 and 6[6,65] but apparently not at 16; in vivo studies in this species appear to be lacking. A careful search for urinary metabolites of injected estrone and estradiol in the golden hamster has revealed[7] only 2-oxygenated steroids. In vivo studies in the guinea pig have indicated[10,66] little or no hydroxylation to occur. However, guinea pig liver slices and microsomes appear to contain active 16α- and 16β-hydroxylases (see

Section II.E). Extensive in vivo studies in the rabbit[8,9] have not provided evidence for estrogen hydroxylation; however, in vitro experiments with rabbit liver slices have demonstrated 2-[67] and 16β-[68] hydroxylation. In the adult dog, 16α-hydroxylation occurs in vivo,[69] with no detectable 2-hydroxylation. Interestingly, the major hydroxy metabolite was 17-epiestriol, with much lesser amounts of estriol. An in vivo study in the newborn canine[70] revealed no 2- or 16-oxygenated compounds, although some evidence was obtained for 15α-hydroxylation. Perfusion of bovine adrenals with estradiol has provided evidence[71] for 15α-, 16α-, and 16β-hydroxylation; ox adrenal tissue is reported[72] to introduce 11β- and 16α-hydroxyl groups into the estrogen molecule. Porcine adrenal tissue apparently contains a 14α-hydroxylase.[73] The rhesus monkey produces[74] 16α- and 16β-hydroxy phenolic steroids, while the baboon produces 16α-hydroxylated forms.[75] Urinary metabolites of injected ^{14}C-estradiol in the pregnant baboon[76] include 2-, 15α-, and 16α-hydroxy compounds. Hydroxylation at positions 2 and 16α has been demonstrated[77] in the pregnant chimpanzee, and the pregnant gorilla is known[78] to excrete estriol in its urine. One nonmammalian species investigated (the domestic fowl) forms 16-hydroxy estrogens, although not as major metabolites. These have been identified in both in vivo[79] and in vitro experiments[80] with liver; the 16β form predominates.

While this review is not exhaustive, it does indicate the complexity of the field concerning hydroxylation. If the function of estrogen hydroxylation was solely detoxification, it is curious that so many hydroxylases occur in some species while few, if any, are to be found in others. It is conceivable that at least some of these hydroxylations are catalyzed by enzymes whose true substrates are compounds other than the naturally occurring steroids. It should not be overlooked, however, that recent evidence indicates the occurrence[81] of a multiplicity of cytochrome P450s differing in specificity for various positions of the steroid molecule. Moreover, the effect of various agents, stimulatory or suppressive, upon neutral steroid hydroxylase systems has been shown[82] to differ considerably from that toward certain drug-metabolizing enzymes. Whatever may be the eventual outcome of this situation, it appears that at least the phenolic steroids possessing hydroxyl groups at position 2 or 16α may have an important biological role. For this reason, some biological relationships and interrelationships of these will be expanded upon below.

B. Hydroxylation at 2 and 16α

1. General

Hydroxylation at carbon-16, whether it occurs with a neutral steroid (or steroid sulfate) as substrate prior to aromatization or directly with a phenolic steroid (or sulfate) as substrate, is (in a quantitative sense) mainly associated with hepatic tissue activity. Scattered reports have implicated the ovary[83,84] and placenta[85-87] in estriol formation but the latter, at least, has been seriously questioned.[88] Some 16-hydroxylation has been reported[61,71,72] in the adrenal cortex; one report claims[89] this activity is present in uterine myometrium, although apparently in a small amount. However, hydroxylation at carbon-2 appears to be quite active in a wide variety of tissues such as liver,[5,6,28] placenta,[90-92] prostatic tissue,[93,94] and several parts of the higher CNS,[37,38] in addition to human fetal adrenal[61] and a pheochromocytoma.[95] Such tissue distribution may relate to the nonestrogenic activities of the catechol estrogens (see Section II.A).

Studies involving the catechol estrogens have been complicated by their considerable instability under many experimental conditions. Special precautions have been found necessary[96-98] to yield rational estimates of these steroids. Problems of this type are not encountered to such a degree when dealing with 16-hydroxysteroids, although the ring D α-ketols such as 16α-hydroxyestrone [3,16α-dihydroxyestra-1,3,5(10)-trien-17-one] and, more particularly, 16β-hydroxyestrone [3,16β-dihydroxyestra-1,3,5(10)-trien-17-one] are unstable in some circumstances.[99,100]

2. In Vivo Studies

It is not surprising that much of the information available for 2- and 16-hydroxylation in vivo has been obtained in the human since both pathways are quantitatively important there. Such experimentation, of course, has limitations, and the majority of studies have involved investigation of the steroids — either endogenous or as metabolites of injected radioactive precursors — in blood, bile, or urine. The latter of these was primarily used. Prior to the knowledge that 2-hydroxylation was an important pathway in the human, Fishman et al. concluded[101,102] that estrone, rather than estradiol, was the precursor of estriol. One of these studies[101] involved simultaneous injection of estrone and estradiol, each labeled with a different isotope (^3H or ^{14}C), and measurement of the $^3H/^{14}C$ ratios of purified metabolites in urine and blood with increasing time. The results indicated rapid oxidation of estradiol to estrone (the reverse being slow) with 16α-hydroxylation of the 17-keto compound as shown in Figure 1. This work established the pathway which had already been studied by a number of investigators.[103-105]

With the understanding that 2-hydroxylation in the nonpregnant human transcended even the 16α-hydroxy pathway[2,27] came the knowledge that the hydroxylases for these positions of the steroid appeared to compete for estrone. It was found that the metabolic picture could be quantitatively altered by certain exogenous compounds and was changed in a number of endocrinopathies and disease states. The level of circulating thyroid hormone has a profound effect upon hydroxylation-[106,107] the hyperthyroid state resulting in increased 2-hydroxylation at the expense of the 16α-pathway.[108] The opposite pattern emerged in hypothyroidism. The coincidence of elevated thyroid hormone and catechol estrogen levels is of some interest since both exert a hypocholesterolemic effect. Brown and Strong[107] demonstrated that obese humans were capable of converting exogenous estradiol to estriol more efficiently than were nonobese subjects. The pattern of endogenous urinary metabolites was in agreement with this. More recently, a considerably increased production of urinary 2-hydroxyestrone and a decreased production of estriol from injected labeled estradiol has been reported[109] in women suffering from anorexia nervosa. These findings were opposite to that found for obese women in the same study. The authors suggested that elevated estriol production over an extended period might relate to an increased estrogenic activity[18,21,22] which, in turn, could be involved in the uterine dysfunctions frequently encountered in obesity. However, the increased 2-hydroxyestrogen and decreased estriol production in anorexia nervosa would tend toward an antiestrogenic environment.[40-42] The factors underlying a disordered estrogen metabolism are not understood, and the degree to which this alteration is involved in the etiology of the various clinical abnormalities is not clear, i.e., whether it is cause or effect. It is known, however, that manipulation of thyroid hormone levels results in a relative change in the magnitude of the two hydroxylation pathways.[108]

The relationship between estrogens and breast cancer has prompted a number of studies. The earlier work of Brown[110] showed what appeared to be an increased excre-

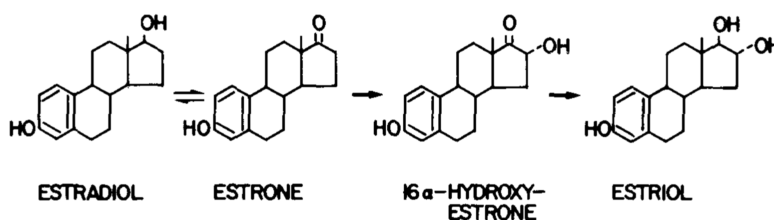

FIGURE 1. Relationship between estrogens and their 16α-hydroxy metabolites in the human.[101,104,105]

tion of urinary estriol, relative to estrone and estradiol, in women with this disease. More recent work[111] has shown no difference in the metabolism of injected labeled estradiol to the various hydroxylated urinary metabolites in women with or without cancer of the breast. The relatively rare male breast cancer has been shown[111,112] to be associated with considerable depression of 2-hydroxylation and increased 16α-hydroxylation. However, another group of investigators was unable to detect this difference.[113]

In human liver cirrhosis, a decreased 2-hydroxylation and a suggested impairment in 16α-hydroxylation has been reported,[114] while in biliary obstruction, some evidence[115] was obtained for a decreased 16α-hydroxylation together with a variable lowering of 2-hydroxylation. It was considered that cholestasis plays a major role in these changes which, interestingly enough, are partially mimicked[116] by administration of the synthetic alkylated steroid norethandrolone (17α-ethyl-10-nortestosterone). Treatment of women suffering from breast cancer with Calusterone® (7β,17α-dimethyltestosterone) has brought about an increase in urinary 2-hydroxyestrogens and a decrease in estriol, both from injected estradiol.[117] Men suffering from benign prostatic hypertrophy reportedly show precisely the opposite picture, i.e., decreased 2- and increased 16α-hydroxylation when administered the antiandrogen cyproterone acetate (6-chloro-1α,2α-methylene-4,6-pregnadien-17α-ol-3,20-dione acetate).[118] In vivo administration of 17α-ethynylestradiol appears[119] to suppress 16α- but not 2-hydroxylation in the human. Two further conditions claimed to be associated with increased 16α-hydroxylation, as reflected in urinary estriol, are prostatic cancer[120] and myocardial infarction.[121]

Despite some controversy over the years, it appears that the pattern of hydroxylated phenolic steroids formed by the nonpregnant human female is similar to that of the male.[27] Moreover, there does not appear to be any major change with age in the adult years. Thus far, no published evidence points to any examples of the congenital absence of the 2- or 16α-hydroxylase.

Catechol estrogens have not been quantitated in urine from nonpregnant humans; however, in pregnancy, the main steroid of this type (in a quantitative sense) is 2-hydroxyestrone.[97,98] This latter compound has been quantitated by radioimmunoassay[122] in both pregnancy and nonpregnancy human blood plasma. The main endogenous 16-oxygenated phenolic steroid in human urine is, of course, estriol.

For many years it was considered that estriol was a steroid metabolite confined to the human. Evidence accumulated during the last 2 decades, however, indicates that 16α-hydroxylase activity, usually demonstrated in vitro, exists in a number of species (see Section II.A). Even so, clear evidence for the in vivo production of estriol has not been obtained in many species and, consequently, little in vivo information has been forthcoming as to the interrelationships of 16-hydroxylated phenolic steroids other than those already referred to above for the human. Such metabolites have been demonstrated to occur in vivo in the chicken and, interestingly, it appears[123] that estradiol (both 17α and β isomers) is the substrate for the 16α- and β-hydroxylases. This is in contrast to the human, where estrone is the substrate.[101,102] (It should be noted that some evidence exists[4] for 16α-hydroxylation of estradiol rather than estrone in rat liver). Despite the considerable number of species in which 2-hydroxylation is known to be quantitatively important (see Section II.A), little in vivo information has arisen in species other than the human as to factors affecting 2-hydroxylase activity.

3. In Vitro Studies

This has been covered to a certain extent in Section II.A and only additional information will be given here. In common with most other hydroxylases, those active at positions 2 and 16 are associated with the microsomal fraction of the cell. Consequently, these have not been highly purified in a soluble form and, therefore, little is

known about their kinetics, specificities, etc. Considerable time elapsed between the initial isolation of estriol[11] from biological sources and the publication of good evidence for its formation under in vitro conditions.[63,124,125] Further work on this subject has mainly involved identifying the steroids formed rather than investigating control of this formation. One exception is the work of Lehmann and Breuer,[126] who noted considerably greater 16- (as well as 6- and 7-) hydroxylation in male than in female rat liver. Steroid treatment of either sex with a variety of steroid types — indicating C_{18}, C_{19}, C_{21}, and synthetic steroids — resulted in stimulation of hydroxylation — implying a lack of specificity. More recently, the relationship of steroid substrate conjugation (mainly the sulfate form) to steroid hydroxylase activity has been under investigation and appears to be leading to a concept of control. This aspect is considered in some detail later in the present chapter.

Despite the wide distribution of 2-hydroxylase, details concerning its properties are lacking, particularly with respect to the form (or forms) present in tissues other than liver. Some studies of the latter have been reported. The rate-limiting reaction leading to formation of water-soluble metabolites of estrogen in rat liver appears to be 2-hydroxylation, which may be controlled by pituitary and steroid hormones[127] and stimulated by spermine.[128] The hydroxylated metabolites may bind to nucleotides, particularly DNA,[129] and this association is not the irreversible type of binding found to occur[130,131] between 2-hydroxyestrogens and protein. A most interesting finding[132] is the presence in the mature rat uterus of a peroxidase enzyme catalyzing the formation of water-soluble metabolites of estrogen. This enzyme can be induced[132] by estrogen and by pregnant mare serum gonadotropin in the immature rat uterus. It has been suggested[133] that the enzyme may be a means of terminating estrogen action in the uterus. The possible relationship of this to steroid hydroxylation is apparent but not yet completely clear.

Investigation of any one hydroxylase may frequently be complicated by the considerable number of such enzymes, leading to multiple product formation, in the same tissue, e. g., rat or mouse liver. Although the golden hamster has been found to exhibit[7] only 2-hydroxylase activity in vivo, no apparent attempt has been made to utilize tissues from this species in an in vitro study. It would seem rational to seek in vitro systems containing as few distinct hydroxylases as possible to more readily study the various relationships, controlling mechanisms, cofactor requirements, etc., of any one system.

C. Steroid Conjugates as Substrates for Hydroxylases

1. Estrogen Glucuronides

All available information points to the formation of estrogen glucuronides as a mechanism geared towards the rapid elimination of the steroidal moiety from an organism. In vivo studies[134] in which labeled forms of estrone or estradiol-3-glucuronides have been injected into humans, followed by analysis of urine, do not in general support the likelihood that the intact conjugates are substrates for hydroxylases. The rapidity of excretion may be too great to allow hydroxylation to occur;[134,135] in addition, it is possible that these highly polar compounds are not readily presented to the enzymes of the endoplasmic reticulum for structural reasons. Formation of estriol glucuronides from estrone or estradiol glucuronide probably occurs via hydrolysis (mainly intestinal) followed by hydroxylation and reconjugation.[136,137] In one study[135] involving injection of estrone-3-glucuronide (which contained ^{3}H in the steroid and ^{14}C in the glucuronyl group) into the umbilical vein during human pregnancy, a small amount of estriol-3-glucuronide of $^{3}H/^{14}C$ ratio equal to that injected was isolated from fetal liver. Thus far, no published attempts have appeared in which pure steroid glucuronides have been exposed to partially purified hydroxylase preparations. It is known that the 3-glucuronides of estrone and estradiol are interconverted both in vivo[134,135] and

in vitro[138,139] without prior removal of the glucuronyl grouping. Also, several isoenzymes of rabbit liver catalyzing the direct oxidation of 17α-estradiol-3-glucuronide at carbon-17 have been considerably purified.[140] Thus, the estrogen glucuronide as a molecule is not entirely uninfluenced by enzymes. However, it should be borne in mind that the above oxidoreductases are almost wholly, if not entirely, soluble in nature.

2. Estrogen Sulfates

It is known that estrone-3-sulfate is a naturally occurring estrogen metabolite present in important amounts — especially in blood,[141-146] but also in other tissues,[57,147] and occasionally in urine.[148] Indeed, it is quantitatively the main estrogen metabolite in the blood of the nonpregnant woman.[142] The human fetus exhibits particular ability[57,149,150] to synthesize estrogen sulfates, and adult human liver slices incubated with labeled estradiol produced estrone sulfate as the main metabolite.[151] Steroid sulfates are conserved by some species, e.g., the human, whose renal and metabolic clearance rates[152,153] are very low compared with those of the glucuronides. This seems to be largely due to considerable binding to serum albumin.[154]

The pioneering work of Twombly and Levitz[155] demonstrated considerable metabolism of ingested ^{14}C-estrone sulfate by the human. Since only one label was used in that study, it was impossible to decide to what degree direct metabolism of the intact conjugate had occurred as compared with that following hydrolysis of the sulfate. More recent work[156] in the same laboratory involved intravenous injection of ^{3}H/^{35}S-estrone sulfate into a woman who had a biliary fistula. Careful analysis of biliary and urinary metabolites revealed a considerable number of 2-, 16-, and 15-hydroxy metabolites. Due to the difficulty involved in separating the various monosulfates from each other in an intact form, it was difficult to state with confidence that hydroxylation of the estrone sulfate had occurred. However, the estriol-3-sulfate formed did contain some ^{35}S, indicating probably some direct conversion. Stronger evidence for direct 2-hydroxylation of estrone sulfate has been obtained[157] in the adult human by administering the ^{3}H/^{35}S form and subsequently recovering estrone sulfate, the 3-sulfate of 2-methoxyestrone [2-methyl-2,3-dihydroxyestra-1,3,5(10)-trien-17-one], and 2-hydroxyestrone-2-sulfate from the urine. The first two conjugates contained 23 and 21%, respectively, of the original ^{35}S while the 2-sulfate of 2-hydroxyestrone contained 7%. This was attributed to direct hydroxylation of the substrate followed by partial O-methylation, on the one hand, and partial conversion of the 2-hydroxyestrone-3-sulfate to the 2-sulfate, with some loss of isotope, on the other. The absence of sulfatase activity in the human fetus[158] has enabled investigators to demonstrate[159] that estrone sulfate is, in these circumstances, the main substrate for 15α- and 16α-hydroxylase activities. Evidence that 2-hydroxysteroid formation may occur via estrone sulfate has been presented for rat[160,161] and monkey[162] liver systems.

These data provide convincing evidence that estrogen sulfates, in comparison with the corresponding glucuronides, can be important substrates for certain hydroxylases. In vivo experiments performed in the human[2,163] have been interpreted to indicate a likelihood that hydroxylated forms of phenolic steroids have their origin in estrone sulfate. In one of these studies, ^{3}H- and ^{14}C-estradiol were administered intravenously and orally, respectively, at the same time.[2] An analysis of urinary metabolites at timed intervals led to the conclusion that a portion of the oral dose was rapidly conjugated in the form of estradiol and estrone glucuronides on the first transit through gut and liver. A further major part of the oral dose mixed with the intravenously administered material and gave rise to hydroxylated metabolites which were excreted in the urine as glucuronides and sulfates. Urinary-conjugated 2-hydroxyestrone, 2-methoxyestrone, estriol, and 16-epiestriol possessed the same ^{3}H/^{14}C ratio as did the 3-sulfates of estrone and estradiol. In a subsequent investigation[163] in which ^{14}C-estrone and ^{3}H-estrone sulfate were intravenously injected into a woman, the isotope ratios of the hy-

droxylated metabolites, regardless of mode of conjugation, were equal to each other and similar to that of the dose. It was concluded that estrone sulfate may very well be the precursor of the hydroxylated metabolites. The widely distributed sulfatase(s) could hydrolyze the hydroxysteroid sulfates thus formed for subsequent reconjugation in the glucuronide form, which is the major type of urinary excretion product in the human.[2,28,148] The possible relationships of sulfurylation and 2- and 16α-hydroxylation are outlined in Figure 2.

Doubt has been expressed concerning the central precursor role of estrone sulfate in estrogen hydroxylation by the human in a publication by Ball et al.[28] Labeled estradiol and 2-hydroxyestradiol were each incubated with liver slices from human males. By far the greater part of the overall hydroxylation occurred at carbon-2 with estradiol as substrate; indeed, metabolism of the two substrates was very similar. Very little 16-hydroxylation occurred, although the possibility was mentioned that prior narcotic treatment of the subjects may have been a factor. It was also noted that catechol estrogen sulfate metabolites were conjugated through the 2-hydroxyl group. The authors reasoned that direct hydroxylation of estrone sulfate would have resulted in the formation of the 3-sulfates of the catechols. In addition, mention was made[28] of unpublished data demonstrating that free estrogens were more efficient precursors of hydroxylated metabolites than were their sulfate forms.

The data from these two laboratories may not be as widely divergent as they appear at first sight. Thus, it has been demonstrated[157] that the 2-hydroxyestrone-2-sulfate excreted in human urine following administration of $^{3}H/^{35}S$ estrone sulfate contained some ^{35}S. This could have arisen via direct hydroxylation of the injected compound followed by hydrolysis and reconjugation at carbon-3 (including some "transfer" of ^{35}S from position 3 to 2). The much greater efficiency noted for hydroxylation of free steroid vs. estrone sulfate by Ball et al.[28] in human liver slices cannot be adequately dealt with here without experimental detail. Although correlation between in vivo and in vitro studies is gratifying, it is not always seen to exist. There can, however, be little doubt that many experimental approaches have demonstrated hydroxylation of free phenolic steroids. At present, the relative quantitative importance of hydroxylation via a "free" vs. "sulfate" pathway is rarely well defined. An exception is the human fetus, where 15- and 16-hydroxylation follows the sulfate pathway, more or less exclusively, in liver tissue, at least.[158] There is no information available as to whether the highly interesting 2-hydroxylase in the tissues of the higher CNS[38,39] utilizes free or sulfated

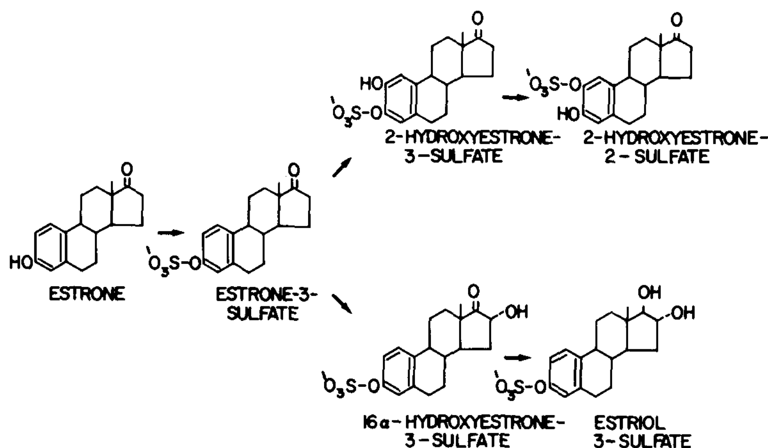

FIGURE 2. Possible scheme for estrone sulfate metabolism in the human with regard to 2- and 16α-hydroxylation.[2,156-158]

estrogen as substrate. Conjugation of estrogen by such tissues has been reported[164,165] although the conjugates formed have not yet been completely characterized. It would be of interest to enquire into the specificity of human placental 2-hydroxylase in view of the overwhelming amount of sulfatase in this tissue.

Notwithstanding the uncertainty regarding the relative importance of the latter two pathways, there can be little doubt that estrone sulfate can be a substrate for certain hydroxylases. In addition, recent investigations[64,166,167] have introduced a further dimension to the picture by demonstrating that prior sulfurylation of the steroidal estrogen molecule results in marked qualitative and quantitative changes in the pattern of hydroxylation. Rat liver microsomes[64] from male animals hydroxylated estradiol at positions 6α, 6β (greatest activity), 7α, and 16α while microsomes from female liver exhibited no such activity. Estradiol-17-sulfate and 3,17-disulfate were not hydroxylated by microsomes from males. Microsomes of female liver, however, formed 6α, 6β, 7β, and 15β hydroxy metabolites from the monosulfate and 7β and 15β hydroxy metabolites from the disulfate. Thus, in the female (but not in the male), there is a hydrophilic species of cytochrome P450. The ability of the male system to hydroxylate the free female hormone may relate to the necessity for inactivation. The same investigators, in a study using human fetal liver microsomes,[166] have demonstrated further specific relationships between hydroxylation and sulfurylation. Unlike the situation with rat microsomes, neither estradiol nor its 17-sulfate was a substrate for hydroxylases. Estrone, on the other hand, was converted to 16α-hydroxyestrone and smaller amounts of 15α-hydroxyestrone. Estrone sulfate was hydroxylated in the 16α-configuration at a rate ten times greater than that of estrone, while 16β-hydroxylation appeared to proceed at a rate similar to 16α-hydroxylation, with the steroid sulfate as substrate. The latter substrate was also subjected to limited hydroxylation at positions 15α and 18. Estradiol-3-sulfate was hydroxylated at 15α and 16α. On the basis of the above data, it was considered that sulfurylation of the substrate had two main effects. First, the sulfate group directs the substrate molecule so that the active site of the P450 interacts with the hydrophobic part of the steroid. Secondly, the sulfate group interacts with the enzyme surface such that a different part of the hydrophobic end of the substrate is exposed to P450 than when the free steroid is involved. It has frequently been suggested that the high level of sulfurylating activity existing in human fetal tissues is a protective mechanism against possibly injurious amounts of highly active hormonal steroids. It has been speculated[166] that the directional influence of the sulfate group on the steroid hydroxylases could lead to the formation of sulfated metabolites which, when subjected to sulfatase action, would result in the release of inactive or poorly active steroids. In a more recent publication[167] dealing with the activity of adult human liver microsomes, it was found that neither the 17-sulfate nor the 3,17-disulfate of estradiol was a substrate for hydroxylases. Estradiol was hydroxylated in the 6α and β configurations, the latter occurring at about seven times the rate of the former. Estrone and estrone sulfate were hydroxylated at 16α with similar rates of formation, while estrone was 6β-hydroxylated three or four times faster than was estrone sulfate. The greatest rate of hydroxylation for any of these substrates in the adult human microsomal system was at 6β for estradiol. When the same hydroxylated metabolite was formed from a steroid sulfate in both fetal and adult systems, its formation proceeded more rapidly in the former. The opposite tended to be true for metabolites of free steroid substrates. Reference was not made to the formation of 2-hydroxy phenolic steroids in any of the studies mentioned above.[64,166,167] Whether these were specifically sought, or whether they were not produced under the experimental conditions, is not clear.

These data demonstrate considerable variation between the three systems (i.e., rat, fetal human, adult human) studied. The presence of a sulfate group in ring D allows hydroxylation by rat liver microsomes but not by those of human origin. Free estradiol

is a substrate for hydroxylases of male rat and adult human livers but not for female rat or fetal human liver. 16α-Hydroxylation is a major activity of human fetal microsomes when estrone, and particularly estrone sulfate, are substrates. In adult human liver microsomes, 6β-hydroxylation is seen to be a major activity.

The interrelationships of the hydroxylases catalyzing hydroxylations at the various positions of the phenolic steroids lead one to conclude that a number of causes could exist for changes in the relative enzymic activities. Thus, for example, certain disease states and endocrine abnormalities, as well as the hormonal environment and some drugs, can bring about, or be associated with, quantitative changes in the pattern of hydroxysteroid metabolites. Such alterations could conceivably also be related to changes in conjugation, particularly with regard to steroid sulfate synthesis. In this respect, cellular sulfatases may be of importance in influencing the amount of free vs. sulfurylated substrates available for hydroxylation. As yet, little is known as to precisely how these controlling factors may function, particularly in vivo. It seems likely that considerable information could be obtained with good model systems. As mentioned earlier, the considerable number of hydroxylases in the tissues of some species can be a complicating factor in the study of any one enzyme. In this author's laboratory, animal systems have been employed in some attempts to further elucidate relationships between free steroidal estrogens, their sulfates, and the hydroxylated metabolites of these. Certain of the results are described and discussed below.

D. In Vitro Studies on Mouse Liver Slices

Although no in vivo experiments on estrogen metabolism in the mouse seem to have been published, in vitro experiments with liver tissue indicate[6,65] the formation of 2- and 6-hydroxylated metabolites from estradiol. Since these latter studies only attempted to identify the lipophilic metabolite fraction, no information is available regarding the conjugated steroids formed. Knowing that hydroxylation does occur, some recent attempts (unpublished) have been made to consider the possibility that mouse liver might form estrogen sulfates which, in turn, could act as substrates for the hydroxylases.

Albino female mice (28 to 32g) were sacrificed by cervical dislocation after which their livers were rapidly excised, washed in cold saline, blotted, and cut into thin slices. Incubations were carried out using 200 mg wet weight of tissue in 10 mℓ Krebs Ringer phosphate buffer,[168] pH 7.4, containing Ca^{++} and 2 mg glucose per milliliter. The substrates were labeled forms of estrogens or estrogen sulfates with specific activities of 28 Ci/mol to 56 Ci/mmol. Incubation was carried out in a Dubnoff® metabolic shaker at 37°C for 1.5 to 2 hr using O_2 or air as the gas phase. The buffer media and slices were then carefully separated, and each was treated with excess methanol prior to filtration and evaporation of the extracts. This was followed by chromatography on Amberlite® XAD-2 resin[169] and on DEAE-Sephadex® (A25) in linear gradients of NaCl.[170,171] Eluted metabolites were analyzed as completely as possible by published methods.[148,170]

Figure 3 shows the Sephadex column elution pattern of radioactivity for tissue and medium from an experiment involving incubation of 4.7×10^6 dpm ^3H-estrone sulfate and 1.5×10^6 dpm ^{14}C-estradiol for 1.5 hr in air. At the termination of incubation, the tissue contained 17 and 18.5%, respectively, of the original ^3H and ^{14}C.

Peak 1 of Figure 3 has not been identified. It was poorly extracted into the usual solvents (e.g., ethyl acetate) and was not hydrolyzed by enzyme preparations such as emulsin. The latter was employed since estrogen glucosides are known[172,173] to be eluted in the position of Peak 1. Peak 2 consisted of 92 to 99% (by crystallization) of a mixture of estrone and estradiol. The ^{14}C content was primarily estradiol, with only a minor degree of dehydrogenation to estrone indicated. The ^3H content, arising from

hydrolysis of estrone sulfate, was almost entirely estrone. Further experiments in which free estrone was the substrate confirmed that little reduction occurred at carbon-17. Although several chromatographic peaks occurred in the "glucuronide" region of the chromatogram (i.e., Peaks 3 to 5 in fractions 20 to 40), no more than approximately 20% of the radioactivity was recovered in organic solvent extracts after β-glucuronidase incubation. Attempts to determine the presence of double conjugates containing both glucosyl and glucuronyl groups did not yield positive information. Thus, sequential incubation with β-glucuronidase and emulsin, or the reverse, led to no additional extractable radioactivity over that released by β-glucuronidase alone. Peak 6 of Figure 3 was identified by crystallization to be estrone sulfate, presumably representing unaltered substrate. The absence of ^{14}C indicated a lack of formation from estradiol; moreover, no estradiol sulfate containing either label could be detected. Peak 7 was the major metabolite to be found in the tissue slice, and it contained both isotopes. Its chromatographic behavior strongly suggested that it was a monosulfate; additionally, it could be extracted into ethyl acetate from a NaCl-saturated solution of pH 6. Less than 20% of the isotopes became extractable into ether after incubation with phenolsulfatase, and it appeared to be only partially recovered following solvolysis. No final identification of this conjugate has been seriously attempted, but it could represent a steroid (or steroids) containing hydroxyl groups at position 2 or 6 and largely conjugated with sulfate in ring B or D.

Since Peak 7 arose both from estradiol and estrone sulfate, it became of interest to enquire whether any of the original sulfate moiety of the latter could be found associated with the new metabolite. ^{3}H-Estrone-3-sulfate-^{35}S was employed as substrate in a series of experiments. Figure 4 shows the chromatographic elution pattern of the isotopes in tissue and medium after incubating 4.6×10^6 dpm of ^{3}H and 1.64×10^6 dpm of ^{35}S in air for 2 hr. At this time, the tissue contained 18% of the incubated ^{3}H and $< 0.5\%$ of the ^{35}S. The main conjugated tissue peak (Peak 7) corresponded to that of Figure 3 and contained only ^{3}H. The steroidal part of this material could have been formed by efficient hydrolysis of estrone sulfate followed, perhaps, by hydroxylation and reconjugation with sulfate from a pool containing little or none of the ^{35}S. No more than a trace of estrone sulfate (Peak 6) remained associated with the tissue slice. The medium contained some residual, doubly labeled estrone sulfate, but by far the major fraction of ^{35}S was present in an inorganic form (fractions 31 to 35). The medium also contained Peak 7 labeled solely with ^{3}H. Additional experiments with ^{3}H-estradiol-3-sulfate-^{35}S as substrate resulted in a considerable amount of Peak 7 in the tissue, accompanied by little or no 3-sulfate of estrone or estradiol. A small amount of doubly labeled substrate was detected in the medium together with even smaller amounts of estrone sulfate containing some ^{35}S. The main "sulfate" peak, however, was Peak 7, labeled only with ^{3}H.

These data do not support the mouse liver slice system as a model for the study of estrogen sulfate formation and hydroxylation of these. The fate of a phenolic steroid 3-sulfate appears to proceed via efficient hydrolysis, and there is little evidence for sulfurylation of the steroidal phenolic group. In addition, the complex metabolism of these steroids in this system, including the uncertain nature of some of the conjugated forms, would make a straightforward study of any individual hydroxylase difficult. This does not, however, imply that the same picture would necessarily hold in all of the various inbred strains of mouse.

E. Studies on the Guinea Pig

1. In Vitro Studies

Published information based upon either urinary and biliary metabolites in the guinea pig in vivo[10,66] or guinea pig liver perfusion studies[174] indicates that conjugation

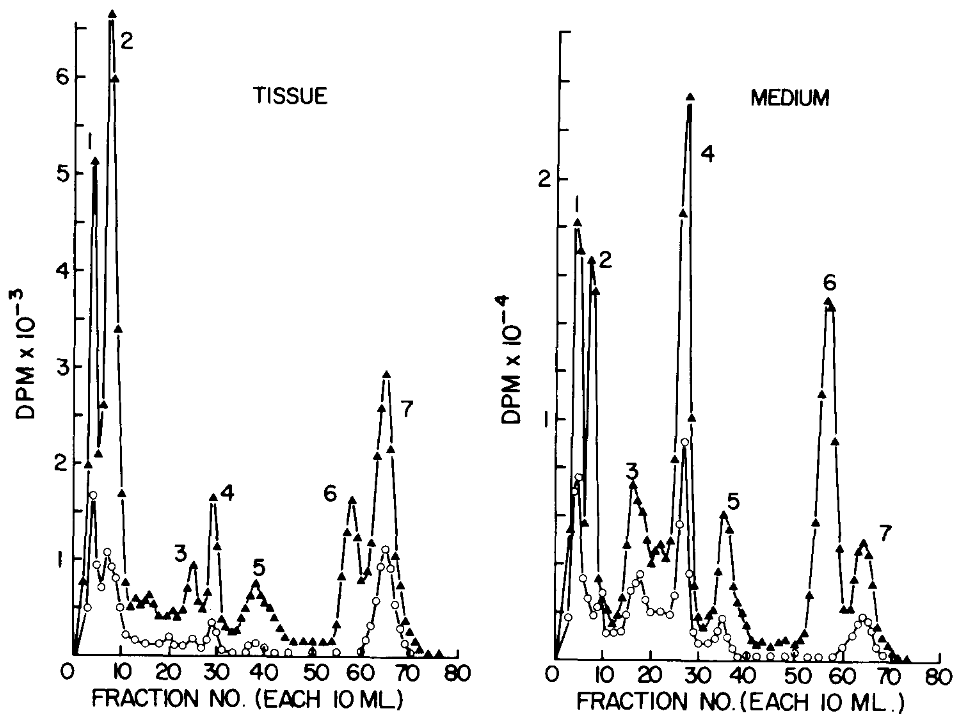

FIGURE 3. DEAE-Sephadex® column elution pattern of metabolites of ^{3}H-estrone sulfate (▲-▲) and ^{14}C-estradiol (o-o) formed by mouse liver slices.

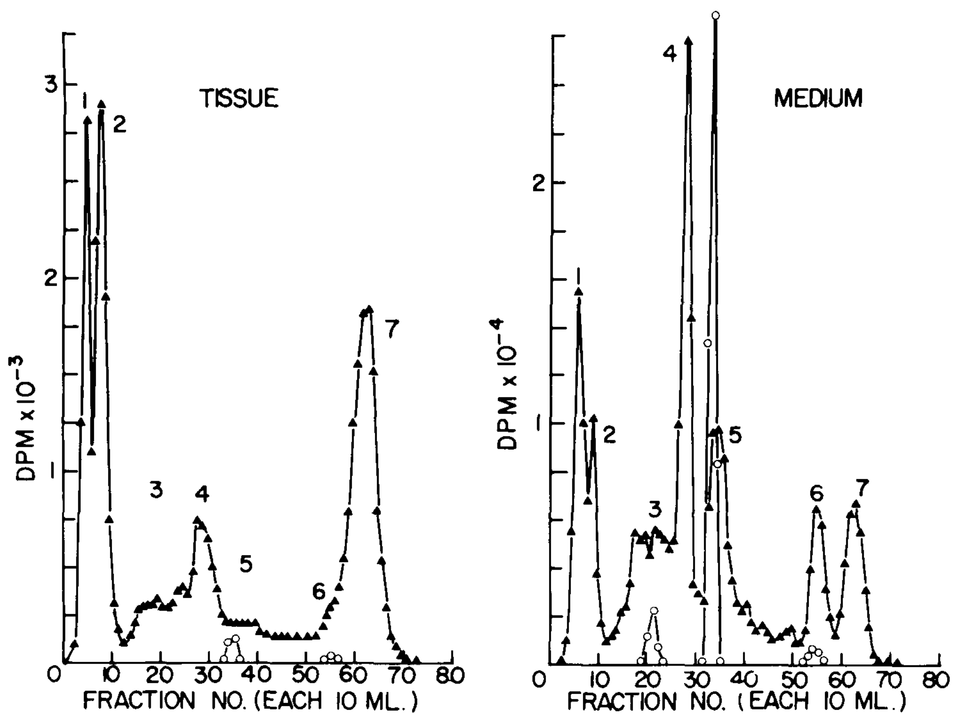

FIGURE 4. DEAE-Sephadex® column elution pattern of metabolites of $^{3}H/^{35}S$-estrone sulfate (▲-▲ = ^{3}H; o-o = ^{35}S) formed by mouse liver slices.

of estrone or estradiol is more or less confined to glucuronide synthesis. Moreover, alteration of the steroid moiety appears to involve little other than oxidoreduction at carbon-17.[10] Nevertheless, it should not be overlooked that guinea pig liver tissue has been widely employed in vitro as a means of forming estrogen sulfates[170,175] as well as glucuronides.[176,177] Guinea pig liver homogenates can form estriol-3,16-disulfate from estriol,[178] and this same transformation can occur in vivo.[179] It has also been reported[180] that placenta from this species can sulfurylate estrone and estradiol. The presence of sulfotransferase activity is thus well established and, together with the reportedly[181] low hepatic sulfatase activity (cf., the rat) suggests the guinea pig as a useful model for study. The recent demonstration[182] that estrone sulfate may be directly hydroxylated in the 16α-configuration further commends the species. Thus, despite apparent discrepancies between certain in vivo and in vitro results, it was considered worthwhile to extend these investigations.

Experiments in which guinea pig liver slices were incubated were similar to those described in Section II.D for the mouse except that 250 mg wet weight of tissue was used in the experiments and incubation time varied from 15 to 120 min. All ^{3}H-substrates were labeled at positions 6 and 7; ^{14}C was at position 4. It was determined that a substrate concentration range of nanomolar to micromolar did not result in any obvious change in the metabolite pattern. Initial studies done on maternal liver slices from animals in the last 2 weeks (50 to 63 days) of gestation revealed[183] that similar overall patterns of metabolites were produced from labeled estradiol, estrone, and estrone sulfate. Little free steroid was found after 2-hr incubations, however, as shown in Table 1, monoglucuronides, monosulfates, and disulfates were isolated. The latter was not estradiol disulfate, but rather, a mixture of hydroxysteroid disulfates whose concentrations in the tissue were much greater than in the medium. It was originally thought that these latter were formed only in maternal liver and not by the nonpregnant or male animal. This was shown to be incorrect, although difficulties have been experienced in demonstrating this disulfate synthesis in liver from albino animals, pregnant or not. Hydroxylation and subsequent disulfate formation proceeds more actively in pigmented animals although even here, the range of results is very wide (see Table 1). The guinea pigs employed were of the English shorthair variety. In all of these studies with liver slices, the recovery of the substrate isotope (^{3}H or ^{14}C) through the various extraction procedures up to the end of the DEAE-Sephadex chromatography averaged 85% (range 78 to 95).

With free steroid as substrate, uptake into the slice was rapid and tissue content remained fairly constant between 15 min and 2 hr. Steroid sulfates were taken up more slowly, but at incubation times of approximately 2 hr, an average of 18% of both free and sulfurylated estrogen labels became associated with the slice.

Figure 5 shows the chromatographic pattern of metabolites found in a maternal tissue slice incubated for 45 min with ^{3}H-estradiol. In this, and all similar experiments, careful analysis demonstrated that Peaks 2 and 3 were composed 88 to 94% of estrone-3-glucuronide and estradiol-3-glucuronide, respectively. Peak 4 was the 3-sulfate of 16α-hydroxyestrone; Peak 5 was estrone sulfate, together with $< 1\%$ of estriol-3-sulfate; and Peak 6 was at least 90% composed of estradiol-3-sulfate. The pattern of metabolites in the buffer media of such experiments was similar to that in the tissues except insofar as conjugated estradiol was relatively more important in amount. This was particularly true of estradiol sulfate and was almost certainly due to considerable sulfurylation of substrate estradiol by soluble enzymes leaking from the tissue. Once within the slice, conjugates of steroids possessing the 17-keto group were predominant. At no time were ring D monosulfates detected. The disulfate peak shown in Figure 5 consists mainly of the 3,16-disulfates of 16α-hydroxyestrone and estriol.[182] Also present are 16β-hydroxylated forms (see further below). The well-defined 16α-hydroxyes-

TABLE 1

Metabolite Pattern in Tissue and Medium Following 2 Hr Incubation of Estrogens and their Sulfates with Female Guinea Pig Liver Slices

				Product (% of isotope in tissue and medium)[b]					
Animals	No. Animals	No. Incubations	Substrate[a]	Free	$E_1$3G	$E_2$3G	$E_1$3S[c]	$E_2$3S	Disulfate
Pregnant	12	14	$E_1$3S	2.7	10.8	0.88	62.2	2.6	18.7
				(0.39—6.0)	(2.5—36.8)	(0.0—4.4)	(48.7—78.4)	(1.3—5.4)	(tr—33.8)
	8	8	E_2	3.9	30.4	13.1	19.6	7.9	21.1
				(0.0—8.7)	(8.9—52.6)	(tr—40.5)	(14.5—33.1)	(1.3—23.9)	(tr—34.3)
Nonpregnant	5	7	$E_1$3S	1.4	8.2	0.40	69.0	3.0	17.4
				(0.3—2.7)	(5.1—11.2)	(0.0—0.82)	(38.3—86.2)	(0.0—4.6)	(tr—47.1)
	5	6	E_2	3.0	36.2	8.5	23.6	7.6	13.0
				(0.0—6.1)	(19.1—44.8)	(5.1—22.1)	(12.3—45.2)	(1.8—13.0)	(1.0—20.4)
	2	2	E_1	2.7	30.5	3.3	47.6	3.6	6.2
				(1.7—3.7)	(25.1—35.9)	(2.6—3.9)	(38.1—57.0)	(2.8—4.1)	(tr—12.4)

[a] Concentration = nanomolar to micromolar.

[b] Mean values, range in parentheses, tr = trace. $E_1$3G and $E_2$3G denote 3-glucuronides of estrone and estradiol. $E_1$3S and $E_2$3S denote the 3-sulfates of estrone and estradiol.

[c] $E_1$3S Peak contains variable amounts of 16α-hydroxyestrone-3-sulfate (see text).

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trone sulfate peak shown in Figure 5 was not evident in all experiments even when major amounts of 16α-hydrosteroid disulfates were present. This might be explained on the basis of different limiting factors in slices from different animals. Thus, depending upon whether ring D hydroxylation or sulfurylation was the rate-limiting step, one might expect to find varying amounts of the 3-monosulfate of 16α-hydroxyestrone. The virtual absence of estriol-3-sulfate in these experiments suggests the pathway; estrone-3-sulfate → 16α-hydroxyestrone-3-sulfate → 16α-hydroxyestrone-3,16-disulfate → estriol-3,16-disulfate. Estriol-3-sulfate could, however, be an intermediate of high turnover rate.

Figure 6 shows the temporal relationships between the various sulfate conjugates with estradiol as substrate in two separate experiments. One should not critically compare these since, in each case, limiting factors would probably be different. However, one can draw the reasonable conclusion that, in this experimental system, estradiol is rapidly sulfurylated to estradiol-3-sulfate which may act as a substrate for estrone sulfate formation. This latter conjugate appears to maintain a fairly constant level over a 2-hr period and probably plays a precursor role in the formation of 16α-hydroxyestrone-3-sulfate and at least part of the disulfate. Some oxidation of estradiol and subsequent conjugation of the estrone formed cannot, of course, be ruled out.

Evidence for direct 16α-hydroxylation of ^{3}H-estrone-3-sulfate-^{35}S has been published[182] and confirmed by further experiments in the author's laboratory.[183] These latter data are summarized in Table 2 and demonstrate direct oxidoreduction between the 3-sulfates of estradiol and estrone as well as direct hydroxylation and disulfate formation.

In Figure 5 Peak 7 (the disulfate fraction) has been subjected to considerable investigation.[182,184] Incubation with phenol sulfatase results in the production of labeled material which behaves on DEAE-Sephadex as a monosulfate (ring D). Solvolysis is difficult and time consuming, and usually some 60 to 70% of the steroid is released in 4 days. Figure 7 shows the elution pattern of the latter material when chromatographed on a 12 × 1 cm Celite® partition column in the system benzene:hexane:methanol:H_2O

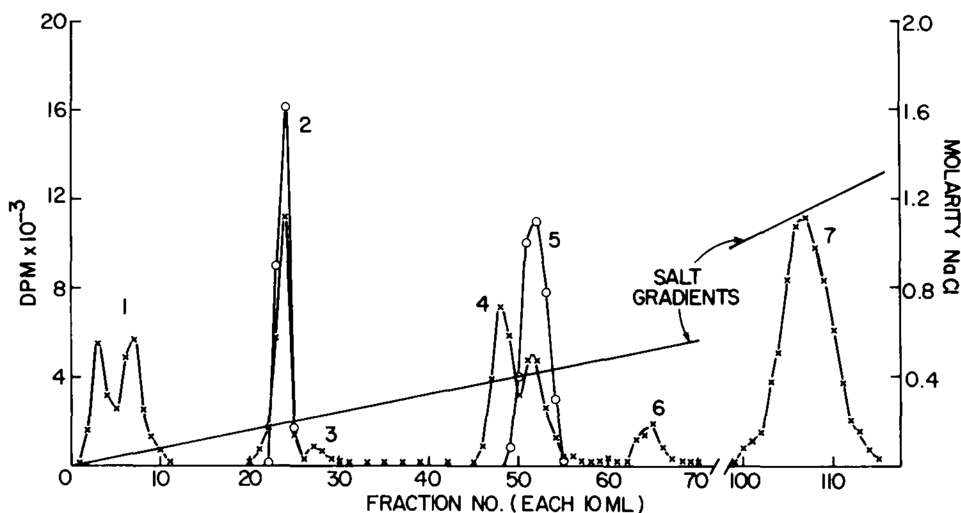

FIGURE 5. Elution pattern from a DEAE-Sephadex® column of labeled metabolites of ^{3}H-estradiol in a pregnant guinea pig liver slice after 45 min of incubation. Peak 1 = free metabolites; Peak 2 = estrone-3-glucuronide; Peak 3 = estradiol-3-glucuronide; Peak 4 = 16α-hydroxyestrone-3-sulfate; Peak 5 = estrone-3-sulfate; Peak 6 = estradiol-3-sulfate; Peak 7 = disulfate; x-x = ^{3}H; and o-o = ^{14}C internal standards. (Reproduced by permission of the National Research Council of Canada from the *Can. J. Biochem.*, Vol. 55, p. 392, 1977).

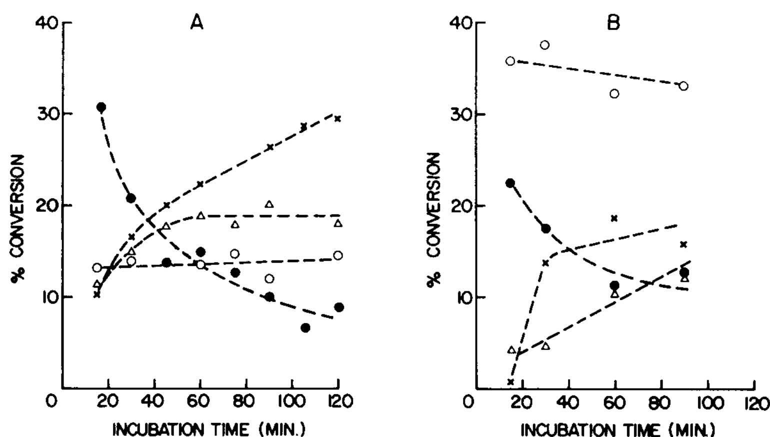

FIGURE 6. Effect of time of incubation on production of metabolites of ^{3}H-estradiol incubated with pregnant (A) and nonpregnant female (B) guinea pig liver slices. ● = estradiol-3-sulfate, O-estrone-3-sulfate; Δ = 16α-hyroxyestrone-3-sulfate; and x = disulfate. (Reproduced by permission of the National Research Council of Canada from the *Can. J. Biochem.*, Vol. 55, p. 394, 1977).

(55:45:70:30) and followed by elution with the upper phase of the system benzene:methanol:H_2O (100:70:30).[185] The column was subsequently stripped with methanol. Material of low polarity eluting in Peaks 1 and 2 has not been identified. Originally, it was tentatively considered[182,183] to consist of Δ^6-estrone and Δ^6-estradiol which could arise via acidification of 6-hydroxylated metabolites. That such is not the case has now been amply demonstrated and it is possible that these peaks arise as artifacts from labile ring D α-ketolic steroids.[184] Peak 3 has been identified to consist of 16α-hydroxyestrone and 16-ketoestradiol-17β. The latter compound may be partly formed during incubation, but at least part of it is almost certainly an artifact arising from manipulation of other ring D α-ketols, primarily 16β-hydroxyestrone. Peak 4 has been identified as 16β-hydroxyestrone by crystallization with carrier steroid as the diacetate and then as 16-epiestriol triacetate formed by reduction of the original steroid and further acetylation.[184] Peak 5, although not a major component of the disulfate

TABLE 2

Isotope Ratios of Products Formed from ^{3}H/^{35}S-Estrone-3-sulfate and Estradiol-3-sulfate[a] during Incubation with Pregnant Guinea Pig Liver Slices

Substrate (isotope ratio)	Incubation time (min)	Isotope ratios of products[b]		
		E_13S[c]	E_23S[c]	Disulfate
E_13S (5.88) Tissue	10—120	6.96 (6.68—7.25)	6.52 (6.28—6.75)	7.20 (6.81—7.75)
Medium	10—120	6.61 (6.45—6.84)	6.38 (5.78—7.00)	7.26 (6.81—7.70)
E_23S (6.47) Tissue	10—120	6.79 (6.30—7.08)	6.41 (6.07—6.86)	6.90 (6.57—7.23)
Medium	10—120	6.10 (5.94—6.25)	6.09 (5.67—6.30)	6.66 (6.45—6.87)

[a] Micromolar concentration.
[b] Mean values (range in parentheses) of three experiments at 10, 30, and 120 min.
[c] E_13S and E_23S denote the 3-sulfates of estrone and estradiol.

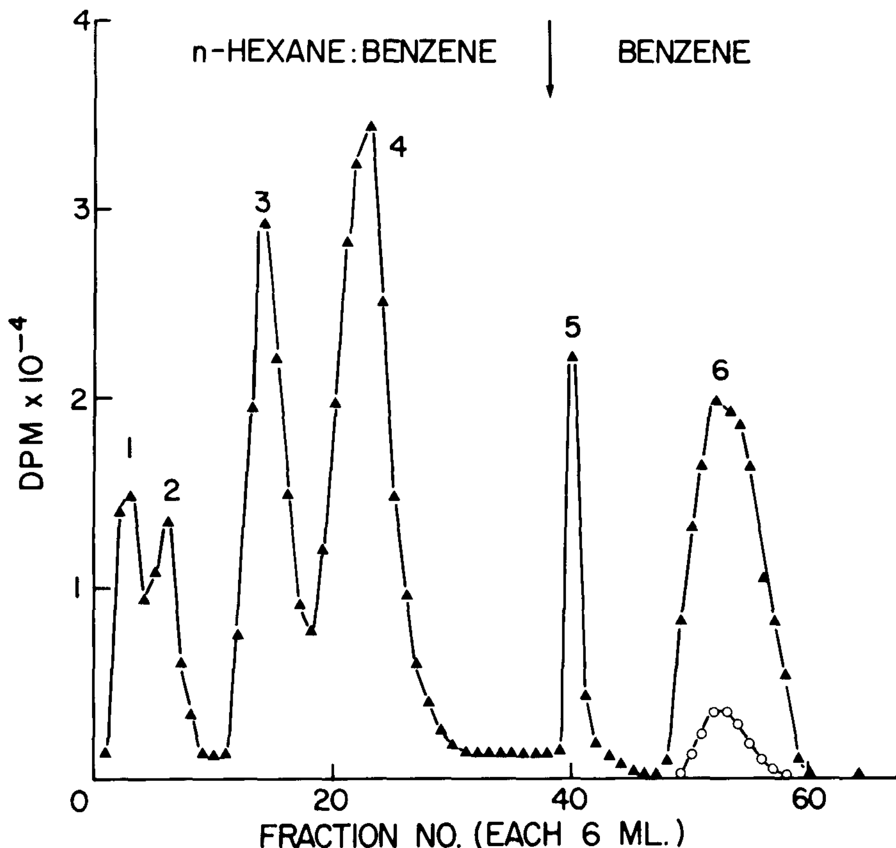

FIGURE 7. Celite column partition chromatography of the solvolyzed ^3H-disulfate fraction formed by incubating ^3H-estradiol with guinea pig liver slices. Peaks 1 and 2, unidentified; Peak 3 = 16α-hydroxyestrone and 16-ketoestradiol-17β; Peak 4 = 16β-hydroxyestrone; Peak 5 = unidentified; Peak 6 = estriol; ▲ = ^3H; and o = ^{14}C from pure ^{14}C-estriol-3, 16-disulfate added before solvolysis.

fraction, is similar to but not identical to 16-epiestriol. It may represent an artifact. Peak 6 was clearly identified as estriol (accompanied by ^{14}C-estriol derived from authentic ^{14}C-estriol-3,16-disulfate added prior to solvolysis). The material eluted with methanol contained ^3H and ^{14}C and behaved on DEAE-Sephadex as a monosulfate. Further prolonged solvolysis indicated that little other than estriol could be released. Therefore, it was concluded that the 3,16-disulfate of estriol is particularly resistant to cleavage, in agreement with the findings of others.[179] Isotope recovery from the partition column was >90%, and it could be estimated that some 60% was hydroxylated in the 16α-configuration and about 28% in the 16β-form. This ignores the approximately 10% of the isotope eluted in Peaks 1 and 2. The same components are detectable in disulfate fractions formed by liver slices from adult animals in which estrone sulfate was employed as substrate, except that the ratio of 16α-:16β-hydroxy metabolites appears to be higher.

In view of the finding that a great variability exists between adult animals with respect to the ability to form the disulfated hydroxysteroids, it was considered feasible to attempt to influence this ability by hormonal treatment of immature animals. However, only a small number of experiments was completed since it soon became apparent that no consistent picture emerged and great variability in disulfate formation was seen in the control animals (Table 3). These 4-week-old guinea pigs were treated daily for 8

TABLE 3

Formation and Identity of Disulfate Formed from ^{3}H-Estrone Sulfate by Liver Slices from Immature Male and Female Guinea Pigs

Animals	Pretreatment	Conversion to disulfate (%)[a]	Composition of disulfate fraction(%)[b]	
			16αOHE$_1$ + E$_3$	16βOHE$_1$
Male	Injection vehicle	10.0, 11.0	83	15
	100 μg E$_2$/day	8.7, 37.0	94	2
	200 μg E$_2$/day	10.7, 15.5	88	12
Female	Injection vehicle	23.0, 24.0	90	n.d.[c]
	100 μg E$_2$/day	17.0, 28.0	84	8
	100 μg testosterone/day	2.8, 17.0	88	9

Note: Two animals were used in each case; the pretreatment period was 8 days.

[a] Individual values.
[b] Based upon a percent solvolysis of 55 to 80%; average values of two experiments in each case. 16αOHE$_1$ = 16α-hydroxyestrone, E$_3$ = estriol, 16βOHE$_1$ = 16β-hydroxyestrone.
[c] Not detectable.

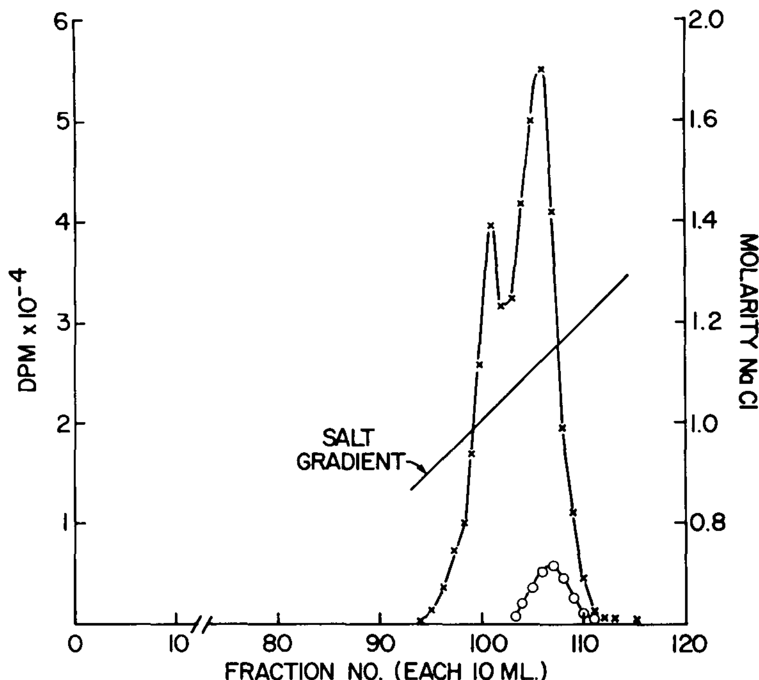

FIGURE 8. Elution pattern from a DEAE-Sephadex® column of labeled disulfate formed by incubation of ^{3}H-estrone sulfate with immature guinea pig liver slices. x = ^{3}H and o = ^{14}C-estriol-3, 16-disulfate. (Reproduced by permission of the National Research Council of Canada from the *Can. J. Biochem.*, Vol. 55, p. 393, 1977).

days with injection vehicle (propylene glycol) alone or with high doses of estrogen or androgen. Liver slices were incubated with ^{3}H-estrone sulfate and the disulfate fraction was isolated. To investigate whether the latter was qualitatively changed by hormone treatment, it was mixed (in each case) with authentic ^{14}C-estriol-3,16-disulfate and subjected to rechromatography on DEAE-Sephadex. A typical elution pattern can be seen in Figure 8 in which a partial separation of two peaks can be detected. Solvolysis of each indicated that the first peak, labeled with ^{3}H, consisted of the disulfates of 16α- and β-hydroxyestrone. The second, contained ^{3}H and internal standard ^{14}C, was virtually all estriol disulfate. Table 3 shows that there seemed to be little difference in the composition of the disulfate fraction following the various treatments, although admittedly, with very small samples. Hydroxylation at 16α was predominant.

Further studies in the author's laboratory[186] employing guinea pig liver microsomal systems have largely substantiated the findings in the slice experiments and have furnished further interesting information. In these investigations, all metabolites were carefully separated and identified employing DEAE-Sephadex chromatography as well as Sephadex LH-20 and Celite partition chromatographic procedures. In addition, when necessary, identification was confirmed by crystallization with appropriate carrier compounds both before and after suitable derivative formation by published techniques.

Simultaneous incubation of ^{14}C-estrone and ^{3}H-estrone sulfate, each in micromolar concentration, for 2 hr with microsomes from adult female animals in a medium containing phosphate, Mg^{++}, K^{+}, Cl^{-}, and NADPH produced the metabolites shown in Table 4. It can be readily observed that the free steroid was hydroxylated primarily in the 16β-configuration, while 16α-hydroxylation occurred with estrone sulfate as substrate. A small degree of 16α-hydroxylation of the free steroid occurred, but virtually no 16β-hydroxylation of the steroid sulfate could be detected. Considerable conversion of the latter substrate to both 16α-hydroxyestrone-3-sulfate and estriol-3-sulfate was evident whereas by far the main hydroxylated metabolite of estrone proved to be 16-epiestriol. Simultaneous incubation of ^{14}C-estradiol and ^{3}H-estradiol sulfate yielded essentially the same metabolites as did estrone and estrone sulfate, respectively, apparently via a rather efficient microsomal steroid 17-dehydrogenase. The glucuronides formed from free steroid substrates were mainly those of estrone and estradiol although that of 16-epiestriol could be detected. Some hydrolysis of steroid sulfate substrates gave rise to estrone and estradiol which were also, at least partially, conjugated in the glucuronide form.

Isotope recovery in a methanol-soluble form following incubation of steroid sulfates was in the 90 to 100% range. However, this fell as low as 60% when free steroids were incubated. The extent of recovery in the case of the free compounds depended upon incubation time (recovery decreased with time) and the presence of NADPH. Consequently, it may be that some metabolite (most likely a hydroxysteroid) is lost through binding to microsomal material, breakdown to a different form, or both. It is not known whether this metabolite is entirely different from the 16α- and β-hydroxylated forms described in Table 4. Conceivably, the labile 16β-hydroxyestrone recovered in rather small amounts after free steroid incubation could be the unaccounted form. It is interesting that when microsomes plus a high-speed supernatant are incubated with estrone or estradiol as above, but with the addition of ATP and SO_4^{2-}, the isotope recovery is 90 to 100%. Rapid sulfurylation occurred under these conditions, and the main hydroxysteroid metabolites were 16α- in character with smaller amounts in the 16β-configuration. Major amounts of disulfates were produced; however, no hydroxysteroids other than 16α and β forms were detected. This suggests that it is one of these, most likely the 16β, which is poorly recovered when free steroid is incubated with microsomes alone.

TABLE 4

Incubation Products of Micromolar Concentrations of ^{3}H-Estrone sulfate and ^{14}C-Estrone incubated for 2 Hr with Adult Female Guinea Pig Liver Microsomes[a]

Products	Conversion from Estrone sulfate	Conversion from Estrone
Estrone	6.7[b] (Estrone through Estriol)	10.6
16α-Hydroxyestrone		2.2
16β-Hydroxyestrone		6.4
Estradiol		3.9
16-Epiestriol		35.2
Estriol		3.4
Total glucuronides (mainly of estrone and estradiol)	1.1	12.8
Estrone-3-sulfate	26.9	7.6[c] (Estrone-3-sulfate through Estradiol-3-sulfate)
16α-Hydroxyestrone-3-sulfate	22.9	
Estriol-3-sulfate	19.7	
Estradiol-3-sulfate	17.3	

[a] Results are the percent of recovered isotope and the means of six incubations with tissue from three animals.
[b] Total free fraction, not further investigated.
[c] Total "sulfate" fraction, poorly defined in chromatographic profile. It may have been formed due to contamination with high-speed supernatant.

Through the use of conditions under which the formation of the 16-hydroxylated products is linear with incubation time, it appears that approximately equal rates of 16α- and β-hydroxylation are catalyzed by the microsomal preparations. The estrone sulfate 16α-hydroxylase exhibits a pH optimum of 7.5 and the Km value has been estimated as 3.6×10^{-5} for estrone sulfate and 4.2×10^{-4} for NADPH. Further studies as to specificity and factors which may control its activity remain to be performed.

2. In Vivo Studies

The rationale for employing estrogen sulfates as substrates in guinea pig systems in vitro and studying 16-hydroxylation of estrogens or their sulfates under the same conditions, would be immeasurably strengthened by evidence in favor of these processes occurring in the animal in vivo. As pointed out earlier in this chapter, most of the in vivo findings in the guinea pig point to glucuronide as by far the major type of conjugate to be found, while oxidoreduction at carbon-17 might be the main (or only) transformation occurring within the steroid molecule. Investigations have, therefore, been undertaken upon the type and tissue distribution of metabolites of labeled estradiol, estrone, and estrone sulfate following injection into mature guinea pigs.

In the first group of animals studied, ^{3}H-estradiol of specific activity approximately 50 Ci/mmol was injected once into each animal. The amount of isotope used varied from 36×10^{6} to 40×10^{6} dpm in each experiment. At times ranging from 1 to 2.5 hr after injection, the animals were sacrificed by cervical dislocation and tissues were rapidly removed. Gall bladder bile and blood were mixed with excess methanol. Liver, kidney, uterus, placenta, and gut were rinsed in cold saline, blotted, and minced before being mixed with excess methanol. All were left at −15°C at least overnight. Methanol extracts, together with washings of precipitated protein where appropriate, were evaporated to dryness in vacuo. Other than for the biliary extracts, the materials were applied to and eluted from Amberlite XAD-2 columns and, where considerable residue

was apparent (particularly with liver), partition was performed in *n*-hexane:methanol:H_2O (100:90:10). The partially purified steroid extracts were subsequently chromatographed on DEAE-Sephadex columns and the eluted peaks identified by procedures variously involving enzyme hydrolysis, Sephadex LH-20 chromatography, Celite partition chromatography, and the usual confirmatory crystallization steps. Table 5 shows the data obtained in a number of experiments. It should be mentioned that the recovery of isotope from the Amberlite XAD-2 column was not always excellent and losses of 10 to 25% occasionally occurred. Consequently, the values in Table 5 (mean values in each case) should not be considered as truly quantitative. In addition, it is conceivable that some radioactivity, nonextractable by methanol, remained associated with tissue residues. Nevertheless, it is felt that the results shown are fairly indicative of the metabolites formed. Estrone and/or estradiol monosulfates were detectable in all tissues examined. A considerable variation in content was seen to exist, but the data clearly support the formation of these conjugates in vivo, although under the experimental conditions they could scarcely be looked upon as major metabolites. Also, the finding of monosulfates in any given tissue does not necessarily imply its formation therein. Thus, although renal tissue contained relatively high concentrations, particularly of estradiol sulfate, there is little evidence for renal synthesis of estrogen sulfates in the guinea pig.[179,187]

Table 6 shows values for labeled metabolites in liver and gall bladder bile in two animals, one at 2 hr and one at 4 hr, after injection of ^{3}H-estrone. Monosulfates were detected and, at both times, small though definite amounts of disulfate were seen in the liver tissue. The bile obtained at 2 hr was devoid of disulfate but that from the 4 hr experiment contained readily detectable amounts. In addition, a peak eluting from Sephadex ahead of estrone glucuronide (see Figure 9) was detected in liver at both times and in the bile at 4 hr. Its identification is not complete, but a portion of it may consist of 16-epiestriol glucuronide (probably the 3- conjugate). Attempts to influence hydroxylation or sulfate formation by the administration of pharmacological doses of estradiol or 17α-ethynylestradiol daily for 9 days prior to labeled steroid injection in several groups of animals (male and female) was unsuccessful. However, these experiments were performed at an earlier stage in the overall study, and the animals were sacrificed 2 hr after isotope injection, perhaps leaving insufficient time in which to detect hydroxylation and subsequent disulfate formation. Thus, the possible effect of exogenous sex hormones upon the in vivo picture remains to be carefully determined.

Table 7 contains results obtained for the tissue distribution of metabolites when ^{3}H-estrone sulfate of specific activity approximately 50 Ci/mmol was injected once into two adult females. At 2 hr postinjection, no evidence could be seen for hydroxylation, however, at 4 hr, definite disulfate levels were observed in bile and liver. 16α-Hydroxyestrone-3-sulfate, as well as an unknown "sulfate" metabolite, were also detected in bile at this time. Disulfate concentration in bile was markedly greater than that in liver although, in the latter tissue, 20% of the total isotope recovered was in the disulfate form whereas the figure was 10% for bile. Isotope recoveries from initial extraction to the end of DEAE-Sephadex chromatography were approximately 90% for liver and bile, but unexplained losses occurred while processing kidney, uterus and blood.

These findings indicated that in vivo hydroxylation took place when estrone sulfate was presented to the tissues and sufficient time was allowed for the process. With this information in mind, an attempt was made to allow a build-up of substrate by injecting three times at ½-hr intervals with sacrifice 5 hr after the initial injection. Two adult females were used for this purpose, one receiving ^{3}H-estrone sulfate (total = 116×10^6 dpm, divided equally over three doses) and the other, ^{3}H-estrone (total = 111×10^6 dpm, similarly divided). Both compounds had a specific activity of approximately 50 Ci/mmol. The methodology was as already described but, in addition, urine was obtained. Insofar as could be determined, all of the urine produced during the 5-hr

TABLE 5

Distribution of Labeled Metabolites of ^{3}H-Estradiol in Guinea Pig Tissues

Animals	Time after injection (hr)	Tissue or fluid (no. of exp.)	Metabolites[a]					Sulfate (%)
			Free	$E_1$3G	$E_2$3G	$E_1$3S	$E_2$3S	
Pregnant (i.v. injection)	1	Kidney (3)	1.97	0.88	2.75	0.15	0.46	9.8
		Liver (3)	0.10	0.19	0.17	0.03	0.05	14.8
		Blood (3)	0.15	0.16	0.07	0.06	0.03	19.1
		Bile (2)	n.d.	39.8	1.06	6.55	n.d.	13.8
		Gut (1)	0.36	2.11	0.62	0.36	0.10	13.0
		Placenta (1)	0.58	0.28	0.12	0.27	0.11	27.9
		Uterus (3)	1.96	0.26	0.05	0.28	0.38	22.5
Nonpregnant females (s.c. injection)	2—2.5	Kidney (4)	4.14	3.77	7.15	0.13	0.26	2.5
		Liver (5)	<0.10	0.74	0.45	0.02	0.01	2.5
		Blood (4)	0.11	0.18	0.09	0.01	n.d.	2.6
		Bile (2)	0.54	85.70	1.76	4.92	0.14	5.4
Males (s.c. injection)	2—2.5	Kidney (3)	2.26	1.11	0.89	0.11	0.27	8.2
		Liver (3)	n.d.	0.37	0.18	0.13	0.08	27.6
		Blood (2)	0.09	0.05	0.02	0.01	0.02	15.8
		Bile (3)	0.20	16.35	0.23	2.70	n.d.	13.9

Note: Mean results (disintegrations per minute $\times 10^{-4}$ per gram wet weight or per milliliter) based upon injection of 40×10^6 dpm of ^{3}H. Gall bladder bile volume is an estimate only.

[a] $E_1$3G and $E_2$3G denote 3-glucuronides of estrone and estradiol; $E_1$3S and $E_2$3S, the 3-sulfates of estrone and estradiol; and n.d., not detectable.

TABLE 6

Distribution of Metabolites of Injected ^3H-Estrone in Liver and Gall Bladder Bile of Female Guinea Pigs

Time after injection (hr)[a]	Tissue or fluid	Metabolites[b]							Sulfate (%)
		Free	[c]	E_13G	E_23G	E_13S	E_23S	Disulfate	
2	Liver	0.03	0.10	0.89	0.31	0.09	0.05	0.08	14.2
	Bile	0.72	n.d.	81.80	2.11	7.12	n.d.	n.d.	7.8
4	Liver	n.d.	0.07	1.03	0.26	0.09	0.04	0.07	12.8
	Bile	1.70	3.15	41.80	1.38	6.25	0.32	0.90	13.5

Note: Values (disintegrations per minute $\times 10^{-4}$ per gram weight or per milliliter) based upon injection of 40×10^6 disintegrations per minute of ^3H. Gall bladder bile volume is an estimate only.

[a] One animal at each time

[b] E_13G and E_23G denote 3-glucuronides of estrone and estradiol; E_13S and E_23S, the 3-sulfates of estrone and estradiol; and n.d., not detectable.

[c] This peak probably contains some 16-epiestriol glucuronide and, in addition, unidentified steroid(s). See Table 8 and Peak 2 of Figure 9.

period was collected, including that remaining in the bladder at sacrifice. It was mixed with excess methanol and filtered prior to chromatography on DEAE-Sephadex. Table 8 contains the data for metabolites in liver, bile, and urine. Recovery of isotope from initial extraction to completion of chromatography was approximately 90% in all cases.

It can be readily seen that considerable 16α-hydroxylation of estrone sulfate occurred. Large amounts of hydroxysteroid disulfate were present, the concentration in urine far exceeding that in bile while the latter, in turn, was greater than in liver. Percentage wise, the disulfate in urine accounted for 68% of total ^3H whereas in bile and liver, respectively, the values were 23 and 46%. Following estrone injection, easily detectable amounts of the chromatographic peak containing 16-epiestriol glucuronide were found, particularly in urine and bile. The chromatographic pattern for the biliary conjugates appears in Figure 9. The presence of some 16-epiestriol glucuronide is in broad agreement with in vitro results (above) in which preferential 16β-hydroxylation of the free steroid could be seen. Nevertheless, as already indicated (also see Footnote C to Table 6), the 16-epiestriol glucuronide is accompanied by presently unidentified metabolites. Urine and bile also contained some 16α-hydroxyestrone-3-sulfate, formed probably after sulfurylation of the free steroidal precursor. Disulfate was present in urine at a concentration 10 times that in bile and 100 times that in liver. As with the estrone sulfate experiment, the percentage contribution of labeled disulfate to the total ^3H recovered was greatest in urine (9.6%), followed by liver (4.6% and bile (0.85%). As expected, even although definite evidence was produced for sulfate and disulfate formation, by far the main products were glucuronides. An unidentified "monosulfate" peak was detected in bile after estrone injection (Table 8 and Peak 7 of Figure 9) that appeared to be similar to the compound(s) already referred to in Table 7. Attempts to hydrolyze this with phenosulfatase were not successful and may indicate either a sulfate group in other than ring A or a labile steroid undergoing breakdown during sulfatase incubation.

3. Current Status of the Guinea Pig Model

Although there are no data readily available regarding endogenous estrogen metabolites in the species, it is apparent from the studies described in Sections II.E. 1 and 2 that guinea pig liver can hydroxylate at carbon-16. Estrone sulfate is hydroxylated primarily, if not wholly, in the 16α-configuration while estrone is hydroxylated mainly at 16β. In vitro and in vivo studies complement each other although much more in vivo work is necessary to establish the findings presented here. The synthesis of estrogen sulfate does occur in vivo. Certain of these findings appear to conflict with those already published by others. One possible explanation could reside in the use of animals of different varieties. The apparently low hydroxylation in albinos has already been referred to. In addition, few if any other studies using preformed estrogen sulfate as substrate have been reported; moreover, the necessity for a sufficient period of time and for substrate build-up to occur prior to the detection of hydroxylation in vivo has perhaps become more obvious in the light of the present studies. With the proviso that confirmatory in vivo experiments must be performed, Figure 10 is considered to represent a reasonable overall scheme for the interrelationships of conjugation and hydroxylation in the guinea pig based upon both types of studies.

The amount of free estrogen available for direct hydroxylation or for hydroxylation following estrogen sulfate formation is severely restricted by the glucuronyltransferase action which removes potential substrate steroid by formation of estrone and estradiol glucuronides. Thus it may be claimed, and rightly so, that the degree of hydroxylation normally occurring in the guinea pig in vivo is quite limited. However, it seems likely that, where continuous production of estrogen takes place, some hydroxylation of steroids (and their sulfates) will be ongoing. Whether these hydroxy metabolites have any

TABLE 7

Distribution of Metabolites of Injected ^{3}H-Estrone Sulfate in Adult Female Guinea Pigs

Time after injection (hr)[a]	Tissue or fluid[b]	Total ^{3}H in methanol extract (dpm $\times 10^{-6}$)	Metabolites[c]							
			Free	$E_1$3G	$E_2$3G	16αOH$E_1$3S	$E_1$3S	[d]	$E_2$3S	Disulfate
2	Liver	1.2	n.d.	0.35	0.22	n.d.	3.1	n.d.	2.5	n.d.
	Bile	0.12	n.d.	6.08	n.d.	n.d.	42.4	n.d.	n.d.	n.d.
	Kidney	0.59	0.46	0.55	2.18	n.d.	0.91	n.d.	8.17	n.d.
	Uterus	0.07	1.09	n.d.	n.d.	n.d.	3.13	n.d.	n.d	n.d
	Blood	0.14	n.d.	0.04	0.03	n.d.	0.90	n.d.	0.17	n.d.
4	Liver	0.20	n.d.	0.15	0.09	n.d.	0.07	n.d.	0.05	0.09
	Bile	0.47	n.d.	14.0	n.d.	1.31	5.38	2.38	±	2.51
	Kidney	0.27	0.13	0.99	1.58	n.d.	0.07	n.d.	0.45	n.d.

Note: Values (disintegrations per minute $\times 10^{-4}$ per gram wet weight or per milliliter) based upon injection of 40×10^6 dpm of ^{3}H. Gall bladder bile volume is an estimate only.

[a] One animal at each time.
[b] Isotope content of uterus and blood at 4 hr was too low for analysis.
[c] $E_1$3G and $E_2$3G denote 3-glucuronides of estrone and estradiol; $E_1$3S and $E_2$3S, the 3-sulfates of estrone and estradiol; 16αOH$E_1$3S, 16α-hydroxyestrone-3-sulfate; and n.d., not detectable.
[d] Unidentified as yet; corresponds to similar material in Table 9.8 and Peak 7 in Figure 9.9.

TABLE 8

Distribution of Metabolites of ^{3}H-Estrone sulfate and ^{3}H-Estrone in Each of Two Adult Female Guinea Pigs 5 Hr after the First of Three Half-hourly Injections

Injected material	Tissue or fluid	Total ^{3}H in methanol extract (dpm $\times 10^{-6}$)	Metabolites[a]								
			Free	[b]	$E_1 3G$	$E_2 3G$	$16\alpha OHE_1 3S$	$E_1 3S$	[c]	$E_2 3S$	Disulfate
^{3}H-Estrone sulfate	Liver	1.47	0.06	n.d.	0.23	0.12	1.89[d]		n.d.	1.17	3.09
	Bile	0.63	n.d.	n.d.	5.37	n.d.	21.26	16.9	n.d.	0.39	12.79
	Urine	44.7	n.d.	n.d.	5.35	3.90	14.66	12.72	n.d.	0.63	80.14
^{3}H-Estrone	Liver	0.70	0.02	0.11	1.23	1.15	n.d.	0.08	n.d.	0.08	0.13
	Bile	5.02	1.03	8.36	126.7	5.73	2.73	3.05	2.23	n.d.	1.30
	Urine	15.0	1.43	13.36	63.7	47.2	2.42	1.62	n.d.	0.39	13.82

Note: Values (disintegrations per minute $\times 10^{-4}$ per gram wet weight or per milliliter) based upon injection of 40×10^6 dpm. Gall bladder bile volume is an estimate only.

[a] $E_1 3G$ and $E_2 3G$ denote 3-glucuronides of estrone and estradiol; $E_1 3S$ and $E_2 3S$, 3-sulfates of estrone and estradiol; $16\alpha OHE_1 3S$, 16α-hydroxyestrone-3-sulfate; n.d., not detectable.

[b] These contain some 16-epiestriol glucuronide and, in addition, unidentified steroid(s); see Table 6 and Peak 2 in Figure 9

[c] Presently unidentified; corresponds to similar material in Table 7 and to Peak 7 in Figure 9.

[d] Mixture of $16\alpha OHE_1 3S$ and $E_1 3S$ poorly resolved in this instance.

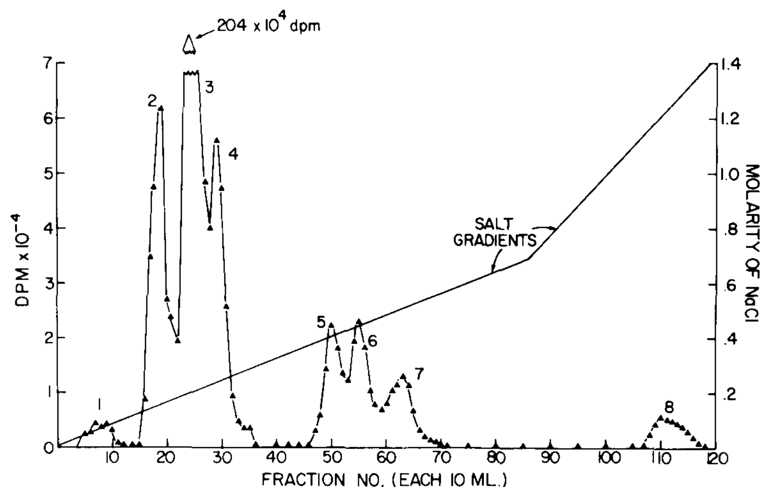

FIGURE 9. Elution pattern from a DEAE-Sephadex® column of labeled biliary metabolites of ³H-estrone injected three times at half-hourly intervals into an adult female guinea pig. The animal was sacrificed 5 hr after the first injection. Peak 1 = free; Peak 2 = partially 16-epiestriol glucuronide, partially unidentified; Peak 3 = estrone-3-glucuronide; Peak 4 = estradiol-3-glucuronide; Peak 5 = 16α-hydroxyestrone-3-sulfate; Peak 6 = estrone-3-sulfate; Peak 7 = unidentified; and Peak 8 = disulfate.

real role to play in the guinea pig is not known. Allowing for this uncertainty, it may be claimed that the guinea pig is a useful model for 16α-hydroxylase investigation. Information on many aspects of this enzyme is sparse despite its major role in estrogen metabolism in the human. It is also worth noting that the two main conjugating mechanisms for steroids which are known to exist in the human, (i.e., sulfurylation and glucuronidation) are also those of the guinea pig. Thus, the latter species may be particularly useful as a means of studying certain aspects of estrogen metabolism as they relate to the human.

Figure 10 assumes that steroid-3-sulfates are the precursors of disulfates in the guinea pig system, and there is evidence in favor of this in the in vitro findings reported in Section II. E.1. However, the possible intermediary formation of ring D monosulfates has not been entirely ruled out; further studies are required on this point. Unpublished data from the author's laboratory has demonstrated that a 3-sulfate group is not mandatory for further sulfurylation of ring D since the 3-methyl ether of estriol is a good precursor, in 105,000 × g supernatants of guinea pig liver, of estriol-3-methyl ether-ring D-sulfate (the exact position of the latter sulfate was not confirmed).

Although 16α- and β-hydroxylated forms of the steroids in question appear to be the major metabolites in the guinea pig, others may be present (see Tables 6 to 8 and Figure 9). The loss of radioactivity which occurs when labeled free estrogen is incubated with liver microsomes (see Section II. E.1) could be due to the formation of additional metabolites which become bound to macromolecules or are transformed to unknown compounds. Repeated attempts in the author's laboratory to demonstrate formation of 2-hydroxy or 2-methoxy metabolites under in vitro and in vivo conditions, have yielded negative results. The good recoveries of metabolites, particularly from in vitro slice incubations, tend to argue against 2-hydroxylation as a major factor in view of the lability of the catechol estrogens. At present, Figure 10 appears to demonstrate the main pathways.

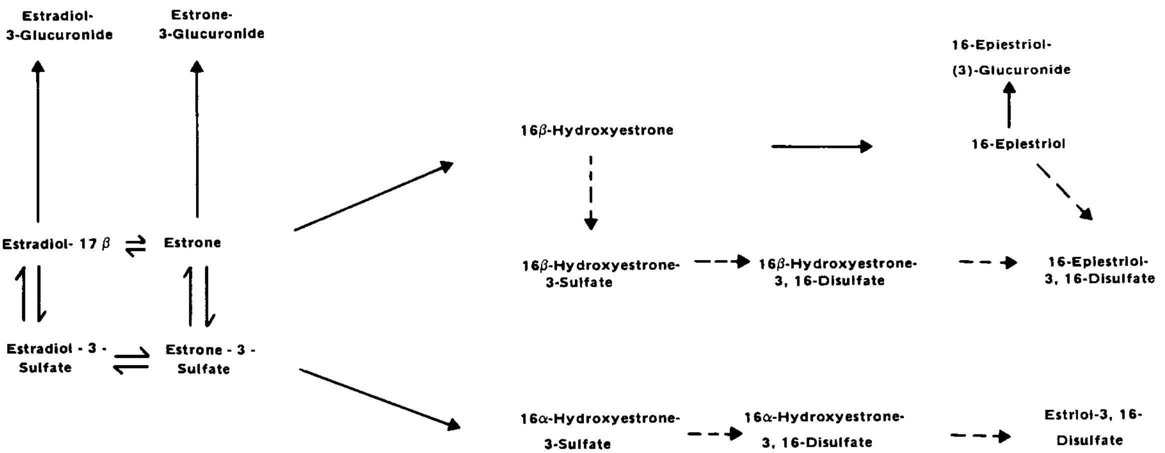

FIGURE 10. Schematic representation of estrogen metabolism, mainly hepatic, as elucidated by in vitro and in vivo experiments in the guinea pig. Broken arrows indicate likely pathways, although some alternatives to these are possible.

F. Summary

A number of sites on the C_{18} phenolic steroid may become hydroxylated, with the pattern varying markedly between animal species. Remarkably little is known about the real significance of such hydroxylations, e. g., whether they represent a complex means of detoxification (inactivation) or may lead to formation of compounds which can exert some biological activity. Evidence is accumulating that 2- and 16α-hydroxy forms may have important functions. Thus, 2-hydroxyestrogens (catechol estrogens) exhibit marked hypocholesterolemic and anticatechol-O-methyltransferase activities. Moreover, under certain circumstances they may be shown to regulate FSH and LH levels or their release. Possible antiestrogenic activity may also be ascribed to this class of steroids. Hydroxylation at 16α can lead to estriol formation which, when continuously produced, may exert estrogenic activity. These two types of hydroxylation, which exist as major pathways in the human and to a greater or lesser extent in some other species, appear to have different tissue distributions. Thus, 2-hydroxylation occurs in liver, placenta, tissues of the higher CNS, adrenal cortex, prostate, and pheochromocytoma of the adrenal medulla. Liver is the major site of 16α-hydroxylation although conflicting opinions exist as to its presence in the placenta and ovary. Some activity appears to be present in the adrenal cortex. In vivo studies in the human show that certain endocrinopathies, administration of natural and synthetic hormones, liver disease and other diseases, as well as a change in nutritional status, may be the cause or result of a marked alteration in the relative degrees of 2- and 16α-hydroxylation. Since such a change could be translated into the formation of a greater or lesser estrogenically active environment, 1, it is important to understand the degree of control which may be imposed upon the hydroxylation processes by the availability of tissue sulfatase(s) and sulfotransferase (or sulfokinase system) enzymes. Many of the model systems available have the disadvantage of forming too many different products thus making it difficult to study any one hydroxylating mechanism. It would be advantageous to use as simple a system as possible, the study of which could result in important new knowledge on the subject of estrogen hydroxylation.

III. C_{19} STEROIDS (ANDROGENS)

A. General

Much of what has been said about the hydroxylated estrogens above appears to be true of the C_{19} neutral steroids. Many of their positions become hydroxylated during exposure to tissues, but the role of the products is even less clear than for the estrogens. There is little knowledge concerning the biological effects of any of the hydroxylated C_{19} neutral steroids. This statement does not, of course, refer to hydroxyl groups at positions 3 and/or 17 in compounds such as testosterone (17β-hydroxyandrost-4-en-3-one), 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and the various diols; the latter were incorporated during biosynthesis or arose by reduction of ketonic forms of the steroids. Despite the lack of information concerning the biological function of the hydroxylated C_{19} steroids, several lines of evidence point to interesting factors associated with the hydroxylases involved. Thus, certain are known to be under endocrine control. Moreover, just as for the estrogens, sulfate conjugation of substrates results in both quantitative and qualitative changes in the pattern of hydroxysteroids produced. It also appears that the origin of at least some hydroxylases is under a genetic type of control. These points, along with other considerations, will be expanded below. Hydroxylation of active androgens is usually associated with a decrease in biological activity but whether this is the primary reason for hydroxylation is not known with any certainty.

B. Types of Hydroxylation and Tissue Sites

Most information has arisen through the use of substrates such as testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one), androsterone (3α-hydroxy-5α-androstan-17-one), and one or more of the isomeric androstanediols — either in the free or sulfated form. Observations have also been made with Δ^4-androstenedione (androst-4-ene-3,17-dione). Depending upon the experimental system employed, hydroxylations are known to occur at carbons 1,2,4,6,7,11,12,15,16, and 18 in one or another tissue such as liver, adrenal, prostate, ovary, skin, and hair follicle. Probably because of the nature and availability of liver from a number of animal species, as well as from midterm human fetus, much of the published work has involved hepatic tissue. At several of the above-mentioned carbon atoms, α- and/or β-hydroxylation has been found.

In rat liver systems, 7α-, 7β-, and 16α-hydroxylation of free dehydroepiandrosterone occurs.[64,188] With the same substrate, 7α-, 7β-, 16α-, and 18-hydroxylation have been demonstrated in hepatic microsomes from human adults[167] and fetuses.[166,189] In addition, human fetal liver homogenates convert dehydroepiandrosterone into its 16α- and 16β-hydroxy forms,[218] while rhesus monkey liver microsomes possess 16α-hydroxylase activity.[190] With testosterone as the substrate, rat liver microsomes hydroxylate at 2α, 2β, 6β and 16α.[64,191] Human liver microsomes (adult[167] and fetal[166]) form 1β-, 2α-, and 6β-hydroxysteroids from this same substrate; the 6β form is particularly important quantitatively in the adult. Mouse liver microsomes hydroxylate testosterone at 1β, 2α, 6α, 6β, 7α, 15α, 15β, 16α, and 16β.[192] Free androstene- 3β, 17β-diol (androst-5-ene-3β,17β-diol) is reportedly hydroxylated at 7α, 7β, and 16α by rat liver microsomes[64] and at 16α by human fetal liver slices.[193] Adult human liver microsomes[167] hydroxylate 5α-dihydrotestosterone at position 4, 5α-androstane-3α,17β-diol at 2 (α and β), 4, 12β, 15α, 16α, and 18; androsterone hydroxylates at positions 6, 16α and 18. A similar, although lower, activity is found[166] in human fetal liver microsomes. Evidence for 16-hydroxylation in rabbit[194] and dog[195] tissue has been reported.

In vitro studies with human fetal adrenals[196] have demonstrated the presence of 1β-, 2α-, 2β-, 6α-, and 6β-hydroxylation of testosterone. 11β-Hydroxylation, not unexpectedly, occurs in the adrenal cortex.[197] Human prostatic tissue showing pathological changes can hydroxylate Δ^4-androstenedione at 2β and 6β.[198] Only 2-hydroxylation was found in normal tissue. The human corpus luteum has been reported[199] to form 6β-hydroxy-Δ^4-androstenedione from Δ^4-androstenedione. Human skin from pubic and abdominal areas has a particular ability to hydroxylate dehydroepiandrosterone at 7α and 7β.[200-202] One of these studies[202] reported 16α-hydroxylase activity in male pubic skin. Human scalp hair follicles convert dehydroepiandrosterone to its 7α-hydroxy form.[203]

Hydroxylation of the angular 19-methyl group poses a special case since many studies[204-206] indicate this to be an essential step in the aromatization process. Although 19-hydroxy intermediates do not normally accumulate under biological conditions, their formation, by inference, occurs in all tissues capable of aromatization. In the human, this includes placenta,[207,208] ovary,[209] testis,[210] fat,[211-213] hair,[214] skin fibroblasts,[215] and possibly liver.[216] In addition, a feminizing adrenal tumor[208] and mammary carcinoma[217] have been shown to possess this activity. In the human fetus, a considerable number of tissues (including liver)[218,219] and those of the higher CNS[219] (including hypothalamus) are able to aromatize C_{19} neutral steroids. This activity is shared by placenta from a number of species including sheep, cow, horse, and sow,[220] armadillo,[221] orangutan,[222] goat,[223] and baboon.[224] In the last species, 19-hydroxy intermediates were detected. Placental tissue of rabbit and guinea pig are reported[220] to lack the aromatizing system. Ovary,[225-227] testis,[228] pineal gland,[229] and tissues of the CNS[230-233] can catalyze aromatization in a number of nonhuman species. Mandibular bone of the rat has also been reported[24] as active in this respect.

All of the hydroxylations referred to in this section, with the exception of mitochondrial 11β-hydroxylation, appear to be associated with the endoplasmic reticulum.

C. Steroid Conjugates as Substrates for Hydroxylases

1. Glucuronides

As in the case of the estrogens (Section II.C.1), little evidence exists to indicate that neutral C_{19} steroids conjugated with glucuronic acid are substrates for hydroxylases. This could be due to the nature of the glucuronyl group which might impose a limitation upon any association between the substrate and particulate enzymes. Such a situation would be in accordance with the direct 5β-reduction of testosterone glucuronide by a soluble reductase without an accompanying 5α-reduction by the endoplasmic reticular 5α-reductase in the human in vivo.[235] The rapid clearance of steroid glucuronides would also interfere with potential glucuronidation. The possible activity of partially purified hydroxylases upon these conjugates does not appear to have been investigated.

2. Sulfates

Considerable interest has centered around dehydroepiandrosterone sulfate, a major circulating compound in the human and particularly in the human fetus. 16α-Hydroxylation of this compound occurs in pregnancy,[236] particularly in the fetal liver (for review of feto-placental relationships, see Reference 57), as an early stage in estriol synthesis. In addition, 19-hydroxylation of this conjugate has been reported in the placenta[237] and ovary.[238] A recent publication[239] has demonstrated 16β-hydroxylation of the same compound in human fetal liver homogenates. Human liver microsomes, adult[167] and particularly fetal,[166] hydroxylate dehydroepiandrosterone sulfate predominantly at 16α whereas the free steroidal form undergoes hydroxylation at 7, 16, and 18. The adult microsomes show much more activity toward the free steroid than toward the conjugate. The latter two publications[166,167] demonstrate the marked effect of sulfurylation upon the hydroxylation of a number of other C_{19} steroids. Thus, testosterone sulfate is hydroxylated at 2β whereas free testosterone gives rise to 1β-, 2β- and 6β-hydroxy forms. Androsterone sulfate undergoes hydroxylation at carbon-18 at a much greater rate than does the free steroid, but the latter is also hydroxylated at 6 and 16α. The sulfate of 5α-dihydrotestosterone, the 17-monosulfate of 5α-androstane-3α, 17β-diol, and the 3,17-disulfate of the latter steroid are not substrates for human liver hydroxylases. This is in contrast to the several positions hydroxylated on the free steroids (see Section III.B). The generally greater activity toward the steroid sulfates on the part of human fetal liver enzymes may be related to the high levels of these conjugates in the fetus. It should be noted that testosterone sulfate is not a substrate for the human placental aromatizing system,[240] a finding which might point to a failure of 19-hydroxylation.

The hydroxylation of steroids and their sulfates differs in some important respects between human and rat liver systems. The most striking divergence concerns the sulfurylated forms of 5α-androstane-3α, 17β-diol.[64,241] The 3-sulfate is not a substrate for rat liver microsomes, whereas the 17-sulfate is hydroxylated mainly at 7β and 15β and, to a lesser degree, at 2α. Differences between sexes have been demonstrated (see Section III.D). The 3,17-disulfate of the latter steroid is specifically hydroxylated at 15β by female rat liver microsomes, but neither the disulfate nor the 17-monosulfate of 5α-androstane-3α,17β-diol is a substrate for human liver hydroxylases.[166,167] It may be noted that 5β-androstane-3α,17β-diol disulfate, unlike the disulfate of the 5α,3α,17α isomer, is hydroxylated at position 15β by female rat liver microsomes. Considerable differences, both qualitative and quantitative, are found in rat liver with regard to hydroxylation of free and sulfurylated steroids.[64] Specifically, no 15β-hydroxylation of a free steroid occurs. A recent publication[81] deals with the recognition of a multi-

plicity of cytochrome P450s in male and female rat livers. These possess some specificity with respect to the carbon atom hydroxylated. For example, three catalyze hydroxylation at 2β and 18; 12β,15α, and 16α; and 15β, respectively. Different P450 species, or binding sites, have also been implicated[242-244] in the aromatization of different substrates by human placental microsomes. Thus, the inhibitory effect of carbon monoxide upon the aromatization of 16α-hydroxytestosterone (16α,17β-dihydroxyandrost-4-en-3-one) is considerable, but upon that of Δ^4-androstenedione is small. A similar variability has been noted during carbon monoxide inhibition of rat liver microsomal hydroxylases.[245]

The available information clearly shows that, as with the estrogens, sulfurylation of C_{19} steroids can markedly affect the amount and type of hydroxylated products formed in biological systems.

D. Regulation and Control of Hydroxylation

Before dealing with the various endogenous factors known to affect hydroxylation of steroids, environmental conditions should be mentioned. Almost all in vitro work done on C_{19} steroid hydroxylation has made use of the smaller laboratory animals, primarily the rat. One particular publication[246] deals with the effect of room lighting upon hydroxylation of dehydroepiandrosterone by liver microsomes from the adult male rat. A reversed light cycle led to increased 7α-hydroxylation whereas constant illumination resulted in low activity. Conditions of constant darkness caused the highest rate of steroid metabolism. These effects of light competed quite successfully with the effects of endocrine ablation upon hydroxylase activity. A further study by Ford et al.,[192] using a mouse liver microsomal preparation for the hydroxylation of testosterone, has demonstrated that the metabolite pattern in the first few weeks after the mice were introduced into the animal room could be quite different from that found beyond 1 month. Such information is of considerable importance and should always be considered in relation to the results obtained.

Evidence has accumulated regarding gonadal hormone control of hepatic 16α-hydroxylation (usually with dehydroepiandrosterone as substrate) in certain species. Moreover, 7α-hydroxylase may also be influenced in this fashion although one report[247] suggests otherwise. 16α-Hydroxylation of dehydroepiandrosterone in slices[188] or microsomes[248] of adult male rat liver is many times higher than that in the female, where it is virtually absent. Castration of the male decreases the activity.[188,246] The enzyme in question appears at puberty and increases thereafter; [249] levels in newborn rats of either sex are extremely low.[250] Exogenous testosterone produces an increase in hepatic dehydroepiandrosterone 16α-hydroxylase to normal levels in prepubertal males provided imprinting by testosterone has preceded it,[251] i.e., neonatal castration markedly suppressed the ability of this enzyme to respond in later life. 7α-Hydroxylation in liver is also reportedly suppressed by castration of adult male rats[246] and requires neonatal imprinting by testosterone.[251] This latter enzymic activity behaves in much the same manner as does 16α-hydroxylase in the male animal with increasing age.[252] In the female liver, 7-hydroxylation decreases from 20 days of age onwards. Neither 16α- nor 7α-hydroxylation is affected in the female liver by castration.[252] It is interesting to note that responses of 7α- and 16α-hydroxylases to various manipulations both in vivo and in vitro (e.g., phenobarbital or aminopyrine treatment, SU-9055 treatment, carbon-monoxide inhibition, detergent inactivation) are entirely different for each enzyme.[82,252] Although the above findings have been reported for the rat, no difference in hepatic microsomal 16α-hydroxylation of dehydroepiandrosterone has been noted[250] between the male and female rhesus monkey. Furthermore, in the latter species, 16α-hydroxylation varies with the stage of development as follows: adult liver > newborn liver > fetal liver,[190] i.e., in the opposite direction to that observed in the human.[218]

Care must therefore be taken to consider the experimental species when attempting to evaluate data relating to endocrine control of these hydroxylases.

Hormones other than gonadal steroids have also been implicated in the control of hydroxylation. Adrenalectomy of adult male rats is reported to increase 7α-hydroxylase activity.[246] The masculine imprinted character of 16α-hydroxylase toward Δ^4-androstenedione and of 2α- and 2β-hydroxylases toward 5α-androstane-3α,17β-diol in liver microsomes from male rats castrated at 14 days of age can be abolished by adrenalectomy plus dexamethasone treatment or by ACTH administration without adrenalectomy.[253] These findings could be due to a glucocorticoid action. It has also been demonstrated that hypophysectomy leads to an overall masculinization of female rat hepatic metabolism with a considerable increase in 2α-, 2β-, 7β-, and 18-hydroxylation of 5α-androstane-3α,17β-diol and in 6β- and 16α-hydroxylation of Δ^4-androstenedione.[254] Moreover, masculinization or feminization with exogenous testosterone propionate or estradiol benzoate, respectively, does not occur in the absence of the hypophysis. This suggests[254] a role for a hypophyseal factor(s).

The ovarian[227] and testicular[228] stimulation of aromatization by FSH and the stimulation of the same process in the placenta[255] and ovary[256] by LH or chorionic gonadotropin suggests that these peptide hormones could be expressing their activities upon the 19-hydroxylation step. Sound evidence for the precise mechanism is not available, but recent studies in both ovarian[257] and testicular[258] systems from the rat strongly indicate that LH is involved in C_{19} steroid formation while FSH has its effect upon aromatization. The control of the latter, as it occurs in other tissues, such as the central nervous tissue and fat, is not understood with any clarity. However, some effect of sex hormones upon aromatization of C_{19} steroids in the rabbit CNS has been reported.[230]

The effects of steroid sulfurylation upon the positions and configurations of hydroxyl groups introduced, and the existence in rat liver tissue of a hydrophilic species of cytochrome P450 (apparently quite different from the bulk hydrophobic P450(s)), have been mentioned. Female rat liver microsomes possess a 15β-hydroxylase system active toward 5α-androstane-3α,17β-diol-3,17-disulfate; indeed, the sole metabolite is the 15β-hydroxy metabolite.[241] This enzyme, undetectable in the adult male, is subject to gonadal control. Its activity in the female is measurable at 20 days of age and rises to adult levels by 40 days. Over the same period, very low initial levels in the male become undetectable. Postpubertal gonadectomy does not influence the activity in either sex, but neonatal castration results in feminized levels in the male, an effect which can be abolished by 1.45 μmol of testosterone propionate when administered on the day following castration. Postpubertal castration of the male followed by estradiol benzoate treatment causes a transient and partially feminized pattern whereas similar treatment of the female, and the use of testosterone propionate rather than estrogen, brings about a suppression of the enzyme. Hypophysectomy reportedly[64] abolishes the 15β-hydroxylase in the female and, in the absence of the hypophysis, no induction of the enzyme is seen following estradiol benzoate treatment of the postpubertally castrated male animal. Interestingly, the enzyme in question is stimulated by in vivo administration of its steroid disulfate substrate.[64]

Recent data[259] favor a genetic origin for certain hepatic steroid hydroxylases in inbred strains of mice. Both qualitative and quantitative variations have been observed, and the results of interbreeding of the strains have further suggested[260] an important genetic influence.

E. Role(s) of C_{19} Steroid Hydroxylations

It is apparent from the above review that hydroxylation of C_{19} neutral steroids can have considerable specificity. This, as well as the existence of a number of control mechanisms — including the action of gonadal, hypophyseal and adrenocortical hor-

mones, sulfate conjugation of substrates, and genetic factors — suggests important reasons for hydroxysteroid biosynthesis. Such control, at least partially, is due to a number of cytochrome P450s differing between male and female of the same species and between different species. As with estrogens, it has been frequently inferred that hydroxylation is a means of androgen inactivation. However, as already mentioned, the reason for the considerable number of hydroxylations known to occur in biology is poorly understood. Also, there is remarkably little known about the biological activities of the hydroxy metabolites. One possible exception might be 16β-hydroxydehydroepiandrosterone (3β,16β-dihydroxyandrost-5-en-17-one) which may play a role[261,262] in a form of hypertension accompanied by low renin levels (low renin hypertension). A more recent publication, however, failed to show[263] abnormal levels of this steroid in low renin hypertension or toxemia of pregnancy. Other investigators have demonstrated either no action[264] of this compound when administered to rats, or a very variable one.[265] In the latter study, some groups of animals responded with a retention of Na^+ and a loss of K^+ but in none of these, whether responding or not, was the steroid in question bound with high affinity to any of the renal steroid receptors. 16α-Hydroxydehydroepiandrosterone (3β,16α-dihydroxyandrost-5-en-17-one) reportedly[266] influences the conversion of glucose to glucitol (thought to be enroute to fructose formation) in human placenta. This steroid showed a slight inhibitory effect when present at about 10^{-6} *M* and strong inhibition at about 10^{-4} *M*. However, dehydroepiandrosterone, while a strong inhibitor at 10^{-4} *M*, stimulated the process somewhat at 10^{-5} to 10^{-6} *M*. The possibility that each distinct hydroxylated steroid may have its own role to play seems remote. Clearly, however, considerably more information will be required to yield definitive answers.

One important function for C_{19} neutral steroids resides in their precursor role for estrogen biosynthesis in the human fetoplacental unit as well as in many other tissues. Indeed, it appears that the activity of 19-hydroxy C_{19} steroids toward sexual differentiation and behavior can be ascribed to the phenolic steroids arising from them.[267] Aside from 19-hydroxylation, which is apparently required for aromatization of the naturally occurring C_{19} steroids, it is known that steroids bearing additional hydroxyl groups at other carbon atoms can be substrates for the aromatizing system. Thus, 16α-hydroxydehydroepiandrosterone is an important precursor of estriol in human pregnancy,[57] and steroids with hydroxyl groups at 7,[268] 15,[269] and 18[270] can be aromatized by human placenta. Some degree of tissue specificity is evident in that, while a number of 16α-hydroxylated neutral steroids can be aromatized by the latter tissue, they may not be substrates for the human ovarian system.[271] Since few of the hydroxylated estrogens are known to possess any particular biological activity, it is difficult to speculate upon the biological significance of aromatization of these latter neutral hydroxylated steroids.

F. Summary

A considerable number of sites of the neutral C_{19} steroid molecule may be hydroxylated by enzyme systems in a variety of tissues and by many species. There is evidence for the involvement of multiple cytochrome P450 types in these hydroxylations and reason to believe that these enzyme systems possess some specificity and are not necessarily identical to those concerned with the metabolism of drugs. Some control of hydroxylation by gonadal steroids has been demonstrated while additional factors such as environment, hypophyseal activity, adrenocortical hormones, sulfate conjugation of substrates, and genetic influences are also known to affect the process. There is little knowledge available as to the biological function of the hydroxy steroids produced, apart from their precursor role in estrogen biosynthesis. Speculation has generally led to the possibility that they may simply be formed as detoxification products

of active androgens. However, the number of carbon atoms which can be hydroxylated may suggest further reasons, yet to be elucidated, for their formation.

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