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Biological Esterification of Steroids

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I. Introduction

A. Obfuscating chemistry and the presumptive steroid acetates

FOR MORE than 150 yr, it has been known that sterols such as cholesterol are esterified with fatty acids in the body (reviewed in Ref. 1). The unique properties that make synthetic alkyl esters of steroids useful as pharmacological agents have been widely known for more than half a century (2–5). Nevertheless, the existence of naturally occurring fatty acid esters of steroids has been recognized only relatively recently. Many possibilities exist as to why fatty acid esters of steroids were not found at the same time that other endogenous steroid metabolites were identified. For example, the extracts from steroidogenic organs were usually saponified (6, 7) or partitioned between solvents to remove nonpolar lipids (8, 9), procedures that also would have destroyed or removed the steroid esters. In addition, steroid esters are not excreted in urine (10), the most accessible human source of steroid metabolites. As radiolabeled steroids became available for metabolic studies, nonpolar metabolites were frequently overlooked because nonpolar impurities are often present in the radioactive tracer or nonenzymatic hydrophobic artifacts may be formed during the incubation.

Biosynthetic studies demonstrated that steroid esters, specifically acetates, can be produced artifactually during the isolation process. The 3-acetate of 5α-androstane-3β,17β-diol was identified in canine prostate and epididymis after arterial infusion of [^{3}H]testosterone but it was not formed enzymatically. The steroid acetate was synthesized by transesterification in the solvent, ethyl acetate, which was used for extraction of the tissue (11). Likewise, dehydroepiandrosterone and 5-androstene-3β,17β-diol (Δ^5-androstenediol) acetates were isolated and definitively identified by gas chromatography/mass spectroscopy (GC/MS) after the incubation of large concentrations of dehydroepiandrosterone (up to 100 μM) with mouse liver homogenates (12). The acetylation appeared to be enzymatic since it did not occur in heat-denatured tissues. However, the reaction was dependent upon extraction of the incubation medium with ethyl acetate. Apparently, an activated intermediate was formed that underwent transesterification with the solvent. Subsequently, it has been shown that biosynthetic acetylation can also result from esterase-catalyzed transesterification of organic acetates added to the incubation (13). Other nonenzymatic reactions that produce nonpolar products have also complicated the detection of biosynthetic esters. When 18-hydroxycorticosterone is incubated with quartered rat adrenals, a nonpolar product is formed (14) that has properties of an ester, *i.e.*, reversible hydrolysis. However, when characterized it was unexpectedly found to be a dimer of aldosterone. Thus, the detection of both endogenous and biosynthetic steroid esters was often concealed by one or more of several confounding variables. Nevertheless, as will be discussed below, there were early signs that nonpolar steroid esters existed but they too were overlooked or misinterpreted.

One of the first reports of the biological esterification of steroids was in 1964 when it was found that a nonpolar ^{14}C-labeled metabolite was formed during incubations of [^{14}C]testosterone with either dimethylbenzanthracene-induced rat mammary tumors, normal mammary glands of the pregnant rat, or a spontaneous mouse mammary tumor (15). This radioactive product was converted back into [^{14}C]tes-

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tosterone by hydrolysis, and it was identified as testosterone acetate by its mobility in several paper chromatography systems, as well as by cocrystallization with added carrier testosterone acetate. The possibility that the isolation process could have been responsible for nonenzymatic synthesis of the testosterone ester was eliminated. While the identification appears to have been carefully done, similar but more recent experiments have shown that testosterone is esterified with fatty acids, not acetate. The misidentification is likely a result of the chromatographic systems then available, which are not nearly as powerful as modern systems, and they probably could not completely resolve other alkyl esters. Furthermore, various alkyl esters often cocrystallize.

Other early studies reported that acetates of corticoids at C-21 are formed biosynthetically during the incubation of [^{3}H]cortisol with homogenates of the brain of the neonatal rat (16). The evidence for enzymatic esterification was definitive. The enzyme was an acyl-Coenzyme-A transferase (acyl-CoA transferase) that showed specificity for natural corticoids since several synthetics such as triamcinolone were esterified at very low rates, if at all (17). Again, however, the identification of the biosynthetic esters as acetates was probably incorrect. Similar studies in rat mammary gland (18) and other tissues of the rat, including brain (19), using modern chromatographic and analytical techniques have definitively identified the nonpolar hydrolyzable corticoid metabolites as fatty acid esters and not acetates. The difficulty in characterizing the corticoid esters is discussed further below.

B. Sterol esters: substrates for the cholesterol side-chain cleavage enzyme

The initial discovery of endogenous steroid fatty acid esters was serendipitous, arising out of experiments that were concerned with another family of steroid esters, the polar sulfate conjugates. In 1963 Seymour Lieberman's laboratory (20) uncovered an interesting and unexpected biochemical pathway. They observed that many of the cytochrome P_{450} steroidogenic enzymes used steroid sulfates as substrates, uncovering a previously unknown adrenal pathway to dehydroepiandrosterone sulfate (20). Like the nonconjugated steroids, polar sulfate conjugates were shown to be converted into C_{21} and C_{19}-steroid sulfates. Pregnenolone sulfate is converted into dehydroepiandrosterone sulfate as well as 17α-hydroxypregnenolone sulfate (21). Later it was found that this "sulfate pathway" extended to the first and critical steroidogenic enzyme, the cholesterol side-chain cleavage enzyme. The amphipathic sterol conjugate, cholesterol sulfate, is converted into pregnenolone sulfate by soluble adrenal extracts (22), and intact mitochondria (23), as well as *in vivo* (24). Cholesterol sulfate is a naturally occurring lipid (25), which is formed by the sulfation of cholesterol in a variety of tissues (26). Although the existence of the latter part of the "sulfate pathway" from pregnenolone sulfate to dehydroepiandrosterone sulfate was known, the enzymatic conversion of cholesterol sulfate into a C_{21} sulfate product was unexpected since even minor modifications of the A ring or its substituents markedly decreases the ability of cholesterol analogs to act as substrates for the cholesterol side-chain cleavage enzyme (27, 28). Consequently, it is surprising that the addition of a bulky and charged sulfate ester would result in an excellent substrate for the enzyme. This unusual specificity of steroidogenic enzymes for sulfate conjugates affords a direct pathway from the C_{27} sterol sulfate to the C_{19} conjugate, dehydroepiandrosterone sulfate.

Experiments designed to study the specificity of the cholesterol side-chain cleavage enzyme led to the discovery that compounds with properties similar to fatty acid esters of steroids served as substrates for this steroidogenic enzyme. Model substrates of several C-3 derivatives of cholesterol were synthesized and incubated with an adrenal mitochondrial enzyme preparation to shed light on the structural requirements that permit cholesterol sulfate to act as a steroidogenic precursor (29). With only two exceptions, these analogs did not undergo significant C-20,22 cleavage. Only cholesterol sulfate and one unusual compound, cholesterol acetate, could serve as substrates. The C_2 ester had been synthesized and tested under the expectation that it would serve as a negative control because it was widely believed that alkyl esters of cholesterol are not substrates for the cholesterol side-chain cleavage enzyme and that cholesterol esters in steroidogenic glands must first be hydrolyzed to cholesterol before they can serve in the steroidogenic pathway. Indeed, one of the first steps in the stimulation of steroidogenesis by tropic hormones is the mobilization of the cholesterol stores by activation of cholesterol esterase (30). Consequently, it was surprising that the 3β-short chain acetate ester of cholesterol could serve as an excellent substrate for cholesterol side-chain cleavage enzyme, producing pregnenolone acetate at about the same rate at which cholesterol is converted into pregnenolone (29).

In light of this finding, several other cholesterol esters of increasing chain length were synthesized and tested as steroidogenic substrates. As the chain length of the acyl groups increased, the rate of C-20,22 cleavage decreased up to C_{18}, cholesterol stearate, at which no appreciable cleavage was observed (Fig. 1). However, cholesterol stearate could act as an inhibitor, which indicated that it was capable of interacting in some way with the cholesterol side-chain cleavage enzyme. This study opened the possibility that some fatty acid esters of cholesterol could serve as intermediates in steroidogenesis. Although it was clear that at least two of the common fatty acid esters are not steroidogenic substrates, the lipid droplets, the hallmark of steroidogenic organs, contain a large variety of cholesterol fatty acid esters (31), many of which could possibly serve as substrates for this putative pathway. More recently, the C-20,22 cleavage of short-chain cholesterol esters has been confirmed with a purified cholesterol side-chain cleavage enzyme, eliminating the necessity of a separate enzyme for these esters (32).

II. Lipoidal Derivatives of Δ^5-3β-Hydroxysteroids in the Adrenal and Ovary

A. Endogenous steroid esters

The enzymatic production of pregnenolone esters by the cholesterol side-chain cleavage enzyme opened the possibility that specific cholesterol fatty acid esters might serve in a previously unknown pathway. The structures of the putative

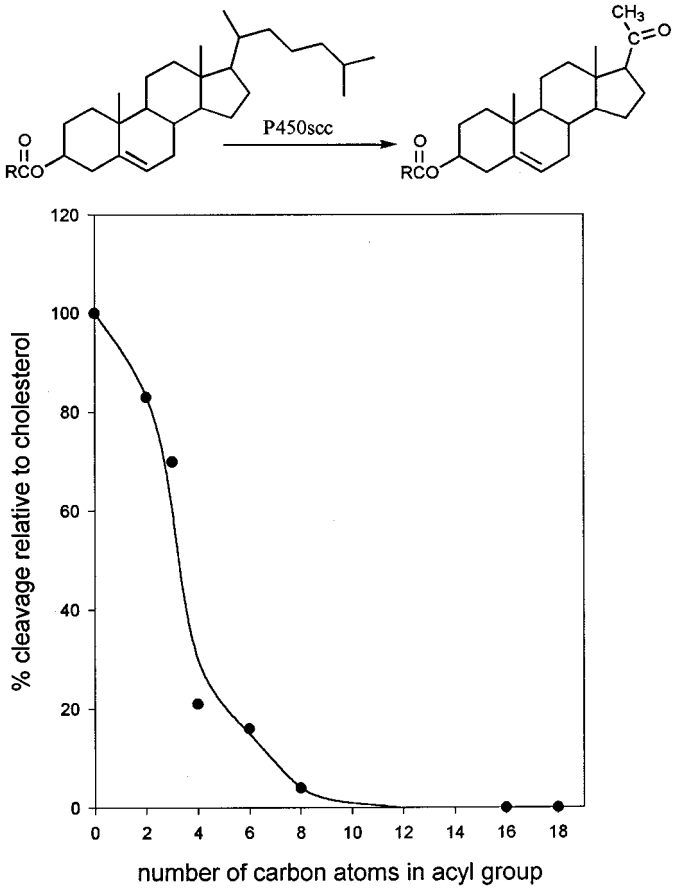

FIG. 1. C-20,22-side-chain cleavage of cholesterol esters by the cholesterol side-chain cleavage enzyme (P450 scc) from bovine adrenals. The number of carbon atoms in the acyl group (n) is = RC=O, *i.e.*, as in cholesterol acetate, n = 2. The rate of cholesterol (n = 0) conversion to pregnenolone is 100%. With the exception of n = 16, which is oleate ($C_{16:1}$), all of the acyloxy groups of the esters are saturated. The figure is derived from data in Ref. 29.

substrates and therefore the specific products, fatty acid esters of pregnenolone, were unknown. Thus, a study was designed to isolate from the adrenal a steroidogenic product with the characteristics of an alkyl ester (33). A nonpolar fraction from an organic extract of bovine adrenal glands was produced and shown by washout experiments with radioactive steroids to contain neither pregnenolone nor polar conjugates of pregnenolone. This nonpolar fraction was treated with either alkali or acid, hydrolytic conditions that cleave esters. The fraction was then analyzed for pregnenolone by acetylation with [^{14}C]acetic anhydride to detect the radioactive product, [^{14}C]pregnenolone acetate, by reverse isotope dilution. This extremely sensitive technique was chosen based on the assumption that any pregnenolone ester present would probably be found in only small amounts. Surprisingly, sizeable amounts of pregnenolone were released. There was almost as much of the nonpolar derivative of pregnenolone (290 μg/kg adrenal) as "free" pregnenolone (435 μg/kg), and there was considerably more nonpolar derivative than pregnenolone sulfate (65 μg/kg). Control incubations without the hydrolytic reagents did not produce pregnenolone. Thus, hydrolysis of the nonpolar fraction uncovered the presence of a previously unknown metabolite of pregnenolone. Based on the amount present in the adrenal, it appeared that a significant pathway had been discovered. It was suspected that the nonpolar pregnenolone metabolite was a fatty acid ester, but the structure then was uncertain. Therefore, the product was named the lipoidal derivative of pregnenolone (PL).[1] The term "lipoidal derivative" is still used to distinguish the entire family of nonpolar steroid esters.

Since it was assumed that a unique steroidogenic pathway had been uncovered, other experiments were performed to search for similar nonpolar metabolites of steroidal products formed in this pathway, including the lipoidal derivatives of other Δ^5-3β-hydroxysteroids, 17α-hydroxypregnenolone (17α-HO-PL) and dehydroepiandrosterone (DL). Lipoidal derivatives of both of these steroids were detected, and the presence of PL was confirmed (34). In this study the ratio of lipoidal derivative to free unesterified steroid was as follows: pregnenolone, 0.41; dehydroepiandrosterone, 0.33; 17α-hydroxypregnenolone 0.1. Thus, all the components of a nonpolar pathway to dehydroepiandrosterone are present in the adrenal. With the possible exception of 17α-hydroxypregnenolone, the lipoidal derivatives are present in sizable amounts well within the same order of magnitude as the free steroid. Bélanger's laboratory (35) confirmed that lipoidal derivatives of the Δ^5-3β-hydroxysteroids are present in the adrenal gland and extended this finding to several species. They detected PL, DL, and the lipoidal derivative of Δ^5-androstenediol. While the lipoidal derivatives are present in smaller amounts than the free steroids (<40%) in most animals, in man they exist in 3-fold greater concentration than their unesterified form (Fig. 2).

Lipoidal derivatives of steroids have also been found in the ovary. A portion of a nonpolar extract obtained from corpora lutea dissected from bovine ovaries and purified by several types of column chromatography was analyzed by GC/MS for the presence of steroids liberated by methanolysis. Both pregnenolone and 3β-hydroxy-5α-pregnan-20-one (allopregnanolone) (36) were observed in the hydrolyzed extract. Mass spectral analysis of the intact ester fraction showed a heterogeneous family of fatty acid esters of the two C_{21} steroids. The endogenous esters of both ovarian steroids were mainly saturated; palmitate and stearate esters accounted for 61% of the total. Later it was shown that homogenates from bovine copora lutea catalyzed the esterification of pregnenolone to PL, but the pregnenolone esters were not enzymatically reduced to allopregnanolone esters (37). When progesterone was incubated with the ovarian homogenate, it was reduced to allopregnanolone, which was then esterified to the lipoidal derivative.

B. Biosynthetic esterification of Δ^5-3β-hydroxysteroids

It was originally suspected that the lipoidal derivatives of the C_{21} and C_{19} steroids found in the adrenal and ovaries

[1] The use of the letter "L" in this review either as a prefix or suffix to name a specific steroid denotes the term lipoidal derivative. Other investigators have used the phrase lipoidal steroid, liposteroid, apolar metabolite, or acyl steroid to name these nonpolar metabolites, which are now recognized as fatty acid esters.

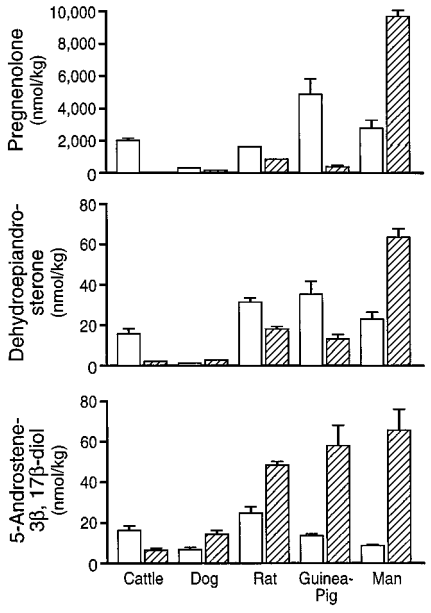

FIG. 2. A comparison of the free and fatty acid esterified Δ^5-3β-hydroxysteroids in the adrenal glands of several species. The *open bars* represent the free unesterified steroids, and the *hatched bars* the lipoidal derivatives. [Reproduced from B. Bélanger *et al.*: *J Endocrinol* 127:505–511, 1990 (35) with permission from The Journal of Endocrinology Ltd.].

were formed in a common biosynthetic pathway in which hydrophobic lipoidal derivatives of steroidal intermediates were the substrates. This hypothetical pathway was thought to originate with a lipoidal derivative of cholesterol. However, another source of these nonpolar metabolites was also possible: one in which each steroid is converted into a lipoidal derivative. To explore the latter possibility, adrenal mitochondrial and microsomal preparations were incubated with dehydroepiandrosterone and pregnenolone. It was found that indeed both were converted into lipoidal derivatives, *i.e.*, nonpolar metabolites that are transformed back into the free steroid under hydrolytic conditions (38). Each of these nonpolar steroidal metabolites was separable into several different components, showing that both PL and DL were heterogeneous metabolites. Subsequently, PL produced biosynthetically by incubation of pregnenolone with adrenal mitochondria was purified by column chromatography (including HPLC) and then analyzed by mass spectroscopy (39). The spectra conclusively proved that PL is a family of fatty acid esters. Five different esters were identified: palmitate, stearate, oleate, linoleate, and arachidonate. Of the five esters, oleate and arachidonate were present in the highest concentration, 42% and 34% of the total, respectively. The fatty acid composition of PL differed markedly from that previously reported for esters of the other major lipids in adrenal mitochondria, indicating that there might be specificity in the esterification of steroids.

Still the source of PL in the adrenal was unknown. It was unlikely that the fatty acid esters of pregnenolone that were identified could have been synthesized by the cholesterol side-chain cleavage enzyme since those esters of cholesterol are not substrates for the enzyme. However, the esters that were identified had been synthesized biosynthetically from pregnenolone, and it was possible that other members of the PL family existed. Another study was performed to isolate the endogenous adrenal PL by using as an internal standard [^{3}H]PL prepared biosynthetically by incubation of [^{3}H]pregnenolone with adrenal mitochondria (34). The biosynthetic [^{3}H]PL, identified as fatty acid esters of pregnenolone (39), was added to an extract of bovine adrenal glands containing endogenous PL. The mixture was chromatographed on a column of Florisil. The fraction eluted from the column was counted to locate the [^{3}H]PL internal standard. Then every fraction was hydrolyzed to analyze for endogenous PL by detecting released pregnenolone by two different techniques: 1) RIA and 2) identification of [^{14}C]pregnenolone acetate after acetylation with [^{14}C]acetic anhydride. Three distinct column fractions contained pregnenolone: only one migrated with the added [^{3}H]PL, while the other two were considerably less polar. Consequently, only one of the three peaks was formed by direct esterification of pregnenolone. It was noted that the Florosil column had very poor resolving power, probably insufficient to separate compounds of similar structure. Thus, it is likely that the PL in the other two fractions is very different than the biosynthetic PL, which consists of several fatty acid esters. The PL in these other two fractions have not yet been identified. It is tempting to speculate that they were formed through C-20,22 cleavage of the corresponding metabolites of cholesterol. Nevertheless, currently there is no evidence supporting such a pathway.

The existence of other pathways involving lipoidal derivatives has been investigated. For example, it was shown that fatty acid esters of pregnenolone are, at best, only poor substrates for the 17-hydroxylase enzyme. [^{3}H]PL isolated from the incubation of [^{3}H]pregnenolone with adrenal mitochondria was incubated with adrenal microsomes in the attempt to detect products of a pathway to C_{19} lipoidal derivatives (40). Although small amounts of 17α-OH-PL and DL were found, their yield was so low that it is unlikely that this pathway is physiologically significant. Similarly, aromatase cannot convert testosterone fatty acid esters into estradiol esters (41). Thus, it appears that steroidogenic pathways utilizing fatty esters of steroids as substrates do not exist.

III. Lipoidal Derivatives of Steroids in Peripheral Tissues

A. Esterification of steroid hormones

Testosterone. In 1973, 4 yr before the discovery of the endogenous lipoidal derivatives of the steroids in the adrenal, it was shown that steroids could be esterified with fatty acids (42). A nonpolar fraction was isolated from a large-scale incubation of the microsomes from two entire calf brains with 40 mg of testosterone and [^{14}C]testosterone. The radioactivity in this fraction was found to migrate with standards of testosterone fatty acid esters and to yield testosterone as well as fatty acids upon saponification. Mass spectral analysis showed the presence of molecular ions consistent with several testosterone esters. Experiments on the *in vitro* esterification of testosterone in the rat indicated that the enzyme was an acyl-CoA transferase, and that it was present in highest concentration in the brain. Furthermore, under the

incubation conditions, brain microsomes esterified testosterone but not cholesterol, indicating that there was specificity in the enzymatic esterification of the C_{19}-steroid. However, these intriguing findings were not pursued further.

Estradiol. The discovery of testosterone esterification and the occurrence of adrenal lipoidal derivatives of the Δ^5-3β-hydroxysteroids increased the likelihood that similar metabolites of other biologically active steroids might also exist. To determine whether estrogens could be esterified, [^{3}H]estradiol (E_2) was incubated with several tissues of the rat. A nonpolar, saponifiable metabolite, the lipoidal derivative of E_2 (LE_2) was detected in many of the tissue extracts (43). The metabolite was found to have properties that are consistent with estradiol esters at C-17: it was saponifiable, resistant to oxidation, able to be acetylated (indicating the presence of a free hydroxyl group), and less polar than estradiol-17-acetate. Since the metabolite was protected from oxidation but had a free hydroxyl, the site of esterification had to be C-17 and not the phenolic C-3 hydroxyl group. This structure was also supported by the finding that estrone, which does not have a hydroxyl at C-17, was not enzymatically converted into a lipoidal derivative.

To characterize biosynthetic LE_2, a relatively large amount of E_2, ~29 mg, was mixed with [^{3}H]E_2 as a marker and incubated with 180 g of bovine endometrial slices (44). The mixture was extracted and an impure nonpolar fraction that weighed 400 mg and contained 0.8 μmol of [^{3}H]LE_2 was obtained by silica gel chromatography. This fraction also contained most of the extracted cholesterol. After subjecting the impure fraction to alumina chromatography followed by reversed phase celite chromatography and three separate HPLC systems, pure LE_2 was isolated. Reversed-phase HPLC separated LE_2 into four distinct fractions. Each of the LE_2 fractions was characterized by mass spectroscopy, which showed a family of E_2 fatty acid esters. The spectra also confirmed that the ester group is at C-17 and not at the C-3 phenolic hydroxyl. The mass spectrum of the predominant ester isolated in this study, E_2 17-arachidonate, is shown in Fig. 3. A portion of each fraction was transesterified and analyzed by GC/MS, which identified 11 different fatty acid esters of estradiol. The fatty acids comprising LE_2 were predominantly unsaturated (86%) and remarkably different from both the cholesterol esters and the phospholipids present in the same tissue extract, another indication that specific enzyme(s) might be involved in the esterification of the steroids.

Abul-Hajj (45) investigated the possibility that LE_2 could be synthesized in breast tumors and thus act as a local source of estrogen. He showed that LE_2 is formed *in vitro* in both rat and mammary tumors (45). When [^{14}C]oleic acid or [^{14}C]stearic acid was included in the incubation, the radiolabeled acids were incorporated into LE_2. This study was confirmed in a number of laboratories. We reported that LE_2 is synthesized in breast cancer specimens regardless of their hormonal sensitivity, *i.e.*, their estrogen receptor and progesterone receptor content (46). The composition of the biosynthetic LE_2 was the same regardless of the steroid receptor content. Adams' group (47) studied the esterification of estradiol in human breast cancer cell lines and noted the formation of LE_2 even at very low, 1 nM concentrations of E_2. They found a biphasic fall in the concentration of LE_2 when the substrate E_2 was removed from the incubations. In further studies (48) the E_2 acyltransferase was characterized in breast cancer specimens and shown to be an acyl-CoA transferase present predominantly in the microsomes. It was confirmed that esterification of E_2 occurs only at C-17. Several C_{19}-steroids, including testosterone, dehydroepiandrosterone, and Δ^5-androstenediol, competitively inhibited esterification of E_2, indicating perhaps that they were all esterified by the same enzyme. However, the inhibition might

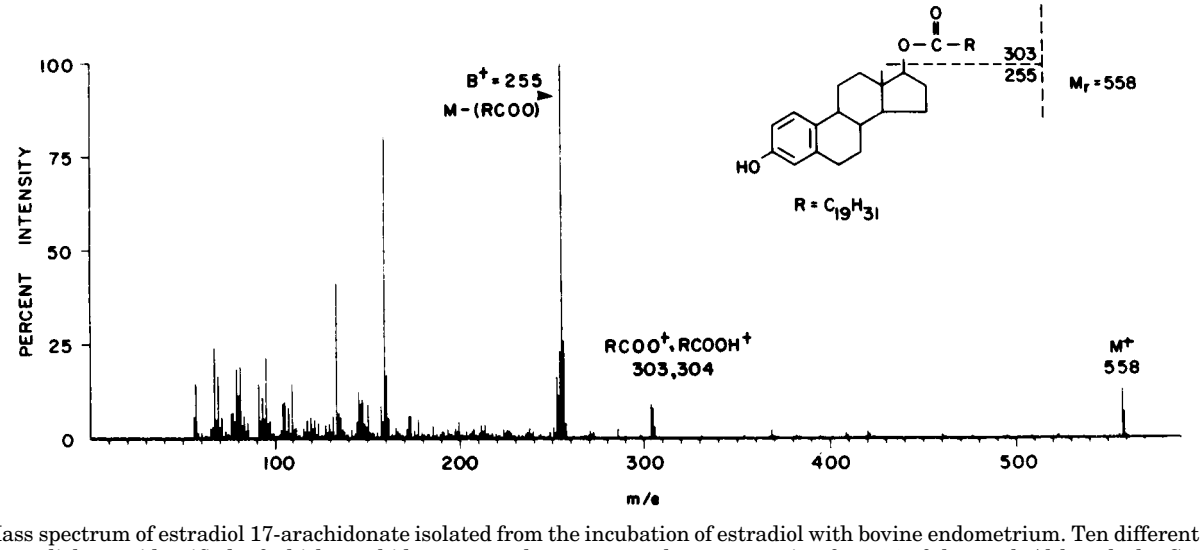

FIG. 3. Mass spectrum of estradiol 17-arachidonate isolated from the incubation of estradiol with bovine endometrium. Ten different fatty acid esters of estradiol were identified, of which arachidonate was the most prevalent, accounting for 27% of the total. Although the C-3 and C-17 esters have the same molecular weight and mass ion (M^+), the fragmentation patterns of the mass spectrum of the two families of estradiol esters are markedly different. Therefore, LE_2 could be characterized as C-17 esters on the basis of the mass spectrum alone. The C-3 and C-17 esters were also easily differentiated by their chromatographic properties [Reproduced with permission from S. Mellon-Nussbaum *et al.*: *J Biol Chem* 257:5678–5684, 1982 (44).]

not have been specific since the K_I of each steroid was of low affinity, in the order of 50 μM. In other studies the esterification of estradiol, as well as of many other steroids, was detected in the human breast cancer cell line ZR-75 (49). The formation of the C-3,17-diester of E_2 was reported. Although we have attempted similar studies with this cell line, we have not detected the formation of the diester.

Lee and Adams (50) found that testosterone is both a substrate for the acyltransferase solubilized from bovine placenta and an inhibitor of the esterification of estradiol, with a K_m of 62 μM and a K_i of 72 μM, respectively. This again indicates that the esterification is not steroid specific. However, inhibition does not assure that both substrates are esterified by the same enzyme, since estrone, which is not esterified, is also a competitive inhibitor in this system (51). Likewise, it had been suspected that the enzyme that esterifies E_2 as well as the Δ^5-3β-hydroxysteroids was probably acyl-CoA:cholesterol acyltransferase (ACAT). In fact, it is a different enzyme (52). Using rat liver microsomes as a model enzymatic system, we found that the esterification of cholesterol and dehydroepiandrosterone (as well as estradiol), all catalyzed by acyl-CoA transferases, leads to steroidal esters with fatty acid compositions different from the cholesterol esters. Furthermore, the ACAT inhibitor, CL 277,082, blocked the esterification of cholesterol almost completely, but had no effect on the esterification of the other steroids. These studies conclusively demonstrated that ACAT is different from the enzyme(s) that esterify the other steroids.

Many different tissues have been shown to synthesize LE_2. We have found that LE_2 is synthesized in various tissues of the rat (19). Lee and Adams solubilized the estradiol acyltransferase from the microsomal fraction of bovine placental cotyledons with sodium cholate (50). The solubilized enzyme was shown to be an acyl-CoA transferase with a Michaelis-Menten constant (K_m) of 8 μM for E_2. They found that a variety of long-chain fatty acid acyl-CoAs could serve as acyl donors and that many had widely varying affinities, with palmitoyl-CoA ($C_{16:1}$) having the highest affinity, K_m of 24 μM and arachidonoyl-CoA ($C_{20:4}$) with the lowest affinity, K_m of 330 μM. Interestingly, the affinity of the acyl-CoA fatty acids do not reflect the percentage of the various esters in LE_2 that have been found in *in vitro* experiments (44, 46, 48, 53) including those utilizing bovine placental microsomes (51), probably indicating that the concentration of the specific acyl-CoA is the most important determinant.

Paris *et al.* (54) developed several reversed phase HPLC systems to characterize LE_2, which they used to study the synthesis of the estrogen esters in bovine liver and adrenal microsomes (53). They confirmed that the enzyme is an acyl-CoA transferase and that the synthesized LE_2 was composed mainly of five esters: arachidonate, linoleate, oleate, palmitate, and stearate. The relative composition of the esters was very different in the two tissues, reflecting the difference in the composition of fatty acids present in the tissues.

Vallet-Strouve *et al.* (55) made a provocative observation about LE_2 biosynthesis in incubations with ovine myometrial cells that they had developed as a model of aging. As the cells were subcultured (aged), the oxidative metabolism of E_2 decreased while the biosynthesis of LE_2 dramatically increased. They interpreted this metabolic change to mean that as the cells aged, they switched over from enzymatic inactivation of E_2 to its preservation through the synthesis of a storage form of estrogen.

Estriol. Estriol (E_3) like estradiol, can be converted into the lipoidal derivative, LE_3. In incubations of [^{3}H]E_3 with rat lung, a tissue that had previously been characterized for LE_2 synthesis (19), we found that 2 radioactive nonpolar metabolites were formed, both having properties of LE_3 (Fig. 4). Further analysis showed that each was a family of D-ring fatty acid esters, one at C-17β (17β-LE_3) and the other at C-16α (16α-LE_3). There was slightly more 16α-LE_3 formed (57%). All of the various esters of both families were separated by reversed-phase HPLC and identified by MS analysis. The percent composition of the fatty acids comprising 16α-LE_3 and 17β-LE_3 was found to be identical and to be the same as LE_2 synthesized in the same tissue, evidence that the esterification of both positions in E_3 as well as E_2 was probably the same. Again, esterification at the C-3 phenolic hydroxyl was not found, nor was a 16,17-diester formed. The dramatic effect of fatty acid esterification on the estrogenic potency of estriol is described below.

Corticoids. While measuring the uptake of [^{3}H]corticosterone by the mammary gland and parametrial adipose tissue of rats, Pearlman's group noticed that some of the ^{3}H-labeled substrate appeared to be acylated (converted into a nonpolar saponifiable metabolite) (56). In a subsequent study they investigated the properties of the nonpolar metabolite and found that it was resistant to periodate oxidation, which showed that it was esterified at C-21. The metabolite was formed in or associated mainly with the nuclear fraction, and it had a polarity between that of corticosterone acetate and butyrate (57). Later, they succeeded in isolating the metabolite and found that it consisted of several fatty acid esters instead of the short-chain ester that had been expected (18). Furthermore, there was another surprise: they isolated one predominant ester, corticosterone 21-oleate, which comprised 80% of the total. A smaller amount of linoleate, 9%, was also found, along with trace amounts of other esters. It is probable that the original experimental results (57), which

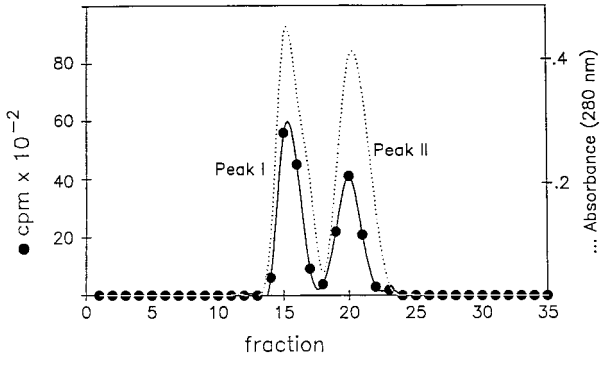

FIG. 4. HPLC characterization of biosynthetic estriol esters (LE_3) as C-16α- (peak 1) and C-17β-esters (peak 11). [^{3}H]LE_3 was isolated from the incubation of [^{3}H]estriol with rat lung. The *dotted line* is the UV absorbance of E_3 16α-stearate and E_3 17β-stearate, which were added to the extract of the incubation medium as internal standards. [Reproduced with permission from S. L. Pahuja *et al.*: *J Biol Chem* 266:7410–7416, 1991 (128).]

seemed to indicate that the corticosterone esters consisted of a more polar short-chain acyl group, were probably caused by oxidation of the fatty acid ester (oleate) to a more polar product during the isolation process. If so, this would also explain why it was originally thought that the corticoids were esterified with acetate (16, 17). Pearlman's laboratory also demonstrated that aldosterone is converted into aldosterone 21-oleate when incubated with rat mammary glands (58, 59). 18-Hydroxycorticosterone, a potential intermediate in aldosterone biosynthesis, is converted into several nonpolar hydrolyzable compounds when incubated with rat adrenals (14). One was identified as a highly lipophilic dimer of aldosterone, and the other appears to be an ester(s) of 18-hydroxycorticosterone.

The biosynthetic conversion of corticosterone into a predominant ester, oleate, was unexpected because the biological esterification of other steroids invariably leads to a complex mixture of fatty acid esters. Consequently, the biosynthesis of LE_2 and the corticosterone esters was compared in parallel incubations (19), and their relative rate of synthesis was found to vary widely in different tissues. It was confirmed that corticosterone is esterified principally to corticosterone oleate (~80%) (Fig. 5, *bottom*) and to other minor esters in rat mammary tissue. However, in the same tissue, E_2 was converted into a heterogeneous mixture of at least nine LE_2-esters (Fig. 5, *top*), a striking contrast to the corticosterone esters. The oleate ester of LE_2 comprised only 18% of the total. In mammary tissue, unusual medium chain (C_8, C_{10}, C_{12}) esters of LE_2 were found, while none were measurable in the corticosterone ester fraction. The esterified 5β-reduced metabolite of corticosterone was also isolated; it too was mainly the oleate ester. The dissimilarity in the composition of the esters of E_2 and corticosterone was reproduced in incubations with uterine tissue. Thus, the enzymatic process that produces LE_2 is different than the one that produces the corticoid esters.

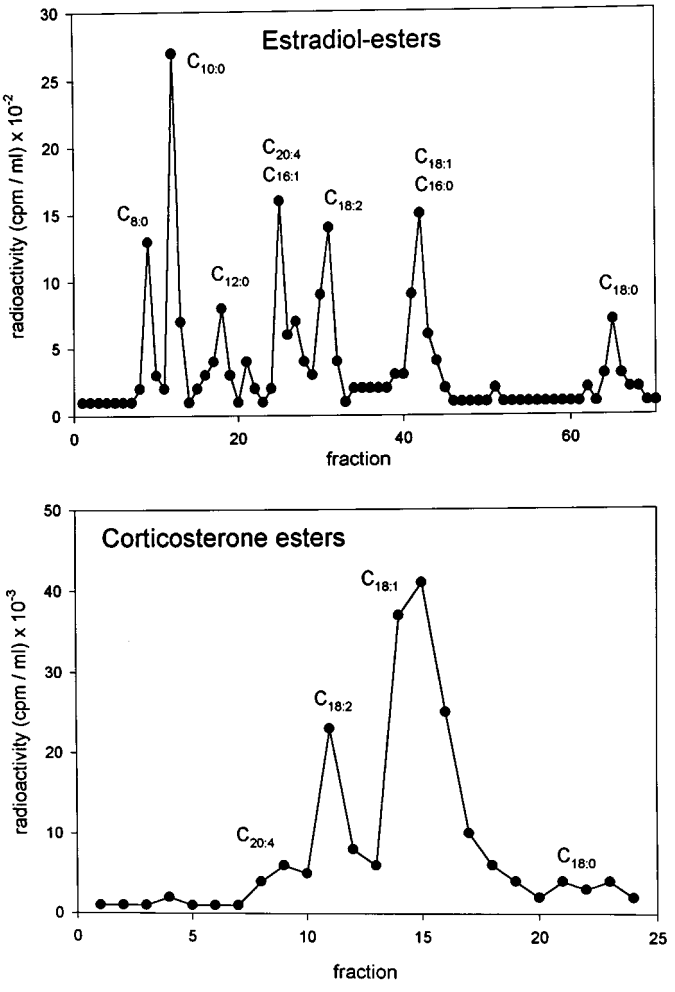

FIG. 5. HPLC of estradiol and corticosterone esters synthesized in rat mammary tissue. The steroid ester fractions were isolated from parallel *in vitro* incubations of estradiol and corticosterone with tissue slices of rat mammary tissue. While the LE_2 fraction (*top*) was resolved into at least nine distinct esters, only four corticosterone esters (*bottom*) were detected of which corticosterone 21-oleate ($C_{18:1}$) approximated 80%. [Reproduced with permission from S. L. Pahuja and R. B. Hochberg: *J Biol Chem* 264:3216–3222, 1989 (19).]

B. Esterification of Δ^5-3β-hydroxysteroids

While it might logically be expected to be androgenic, the 17β-reduced metabolite of dehydroepiandrosterone, Δ^5-androstenediol, has been the subject of considerable interest due to its unusual estrogenic activity (60–62). The possibility that esterification might be a mechanism by which this weak estrogen could be concentrated and thus, increased in potency in tissues, especially breast tumors, led Adams *et al.* (63) to study the formation of the lipoidal derivative of this C_{19}-steroid in breast cells. Incubation of Δ^5-androstenediol and dehydroepiandrosterone with human breast tumor microsomes and various acyl-CoAs led to the formation of lipoidal derivatives of both steroids (63). With Δ^5-androstenediol, equal amounts of two nonpolar metabolites were formed, both of which gave Δ^5-androstenediol upon saponification. Different products resulted if the Δ^5-androstenediol esters were oxidized first and then saponified. The more polar derivative was converted into testosterone, indicating that the 17β-hydroxyl was esterified and that the 3β-hydroxyl was free. The oxidation of the less polar Δ^5-androstenediol ester led to dehydroepiandrosterone, proving that it was a 3β-monoester of Δ^5-androstenediol. Although the microsomal preparation could esterify Δ^5-androstenediol at both C-3 and C-17, the diester was not produced. These C-17 and C-3 esters were also formed when Δ^5-androstenediol was incubated with various human breast cancer cell lines (MCF-7, ZR-75–1, MDA). In the incubations with the cell lines, small amounts of a less polar metabolite was formed, which was thought be the diester. After removal of Δ^5-androstenediol from the cells, the concentration of the esters decreased slowly, and substantial amounts were still present in the cells 24 h later.

Subsequent studies revealed that the ratio of the two types of esters, C-17/C-3, varied markedly in different breast cancer cell lines (64) and that the fatty acid composition of the esters produced in the different cell lines also differed. Other studies of the esterification of Δ^5-androstenediol in ZR-75–1 cells demonstrated that when the incubation medium was removed, most of the radioactive steroid remaining in the cells was esterified (65). Continued incubation in the absence

of added steroid led to a new steady-state concentration of free steroid that was produced by hydrolysis of the esters within the cell. It lasted for a protracted period. These investigators reported that both the C-17 and C-3 ester of Δ^5-androstenediol were formed, but they found that the C-3 hydroxyl was esterified at 5 times the rate of the C-17 hydroxyl. This contrasted with the previous study in which the C-17 ester predominated in this cell line (64). Both studies reported the formation of the diester of Δ^5-androstenediol. However, the diester was not fully characterized except by its mobility in TLC.

A wide variety of steroids including estrogens, glucocorticoids, and androgens in addition to the Δ^5-3β-hydroxysteroids are converted into lipoidal derivatives in the ZR-75–1 cell line (49). 5α-Dihydrotestosterone (5α-DHT) was not esterified although it was converted into 3α- and 3β-reduced metabolites, which were esterified. Testosterone was esterified. In addition, it was metabolized by the 5α-reductase into 5α-DHT, and subsequently converted into C-3 reduced metabolites, which were also esterified. Considering that a variety of steroids, especially testosterone, were esterified in these cells, it seems surprising that 5α-DHT is not a substrate for the acyltransferase. These studies also confirmed that the major ester of E_2 is at C-17β (the C-3 ester was not formed). Traces of a metabolite with the chromatographic mobility of the C-3,17β-diester of estradiol were also detected. Although esterification was not the major route of metabolism, it was noted that the lipoidal derivatives of all of the steroids were the major metabolites present in the cells. These investigators speculated that their prevalence within the cell suggests a key physiological role.

Robel and Baulieu and their colleagues (66) studied the esterification of pregnenolone and dehydroepiandrosterone in rat brain preparations. As expected, most of the acyltransferase activity was present in the microsomal fraction. The biosynthetic PL was isolated and identified as fatty acid esters of pregnenolone (by GC after saponification). The fatty acid composition of PL was compared with that previously reported for the testosterone esters synthesized in rat brain *in vitro* (42). Although there were significant differences in the composition of the esters of these two steroids, the investigators did not determine whether this is due to different enzymes or to different experimental conditions. The PL synthesized in the brain was also compared with the neutral lipids in the preparation; again, the fatty acid composition was significantly different. Several other steroids, including E_2, testosterone, and 5α-DHT, were also shown to be esterified. Interestingly, 5α-DHT is a substrate for the brain acyltransferase but not for the enzyme in breast cancer cells (65). E_2 was esterified at the highest rate of the steroids tested. Corticosterone, 17α-testosterone, and 17α-hydroxyprogesterone were not esterified. The highest levels of acyltransferase activity were found in the 1- to 3-week-old rats. As the animals aged, the activity decreased rapidly. The brains of 14-week-old animals contained only 14% of the enzyme activity in the 3-week-old rat brain. Whether the activity in other tissues also decreased with age was not determined.

Smith and Watson (67) compared the acyltransferase activity in brain, adrenal, kidney, and liver of sheep and rats (at 6 weeks old) using pregnenolone and dehydroepiandrosterone as substrates. Brain tissue in rat, but not in sheep, had the highest activity of the various tissues with both steroids as substrates. In the sheep, the adrenal had the highest activity when dehydroepiandrosterone was the substrate. With pregnenolone as the substrate, the liver had the highest acyltransferase activity, followed by the kidney. Whether this might indicate that there are different enzymes in the tissues or different enzymes for the two Δ^5-3β-hydroxysteroids is not yet known.

C. Endogenous esters of sex steroids

We developed a sensitive GC/MS method to quantify LE_2 (after saponification to E_2) in tissue, using deuterated estradiol stearate as an internal standard for correction of recovery (10). The LE_2 content was measured in several human tissues. The steroid ester was detected mainly in fat, averaging about 250 pg/g in cycling females and about half that amount in menopausal women. There was much less LE_2 in muscle and none in urine. The estrogen ester was also present in blood (LE_2 in blood and ovarian follicular fluid is described below) and in breast cyst fluid, where it averaged about 26 pg/ml, about 10% of the E_2 concentration. Although the concentration of LE_2 in tissues and blood is much less than that of E_2, its striking potency (described below) indicates that it could be a physiologically important store of hormone.

Paris *et al.* (68) investigated the formation of LE_2 *in vivo* after the administration of E_2. They injected large amounts of E_2 (333 mg) mixed with [^{3}H]E_2 into a male calf for 3 days. Shortly after the last injection, the animal was killed and the ^{3}H-labeled metabolites extracted from the perirenal fat were characterized. Approximately 25% of the radioactivity in the fat was present in the nonpolar fraction. Further purification by column chromatography including HPLC showed that the nonpolar metabolite was LE_2. It is likely that, if a longer period had elapsed between the last injection and the analysis of the fat, most of the radioactivity would have been in the form of LE_2. The investigators speculate that the presence of these long-lived steroids in fat could be used as a marker for the detection of estrogens a considerable time after estradiol is administered.

Spurred on by the assumption that testosterone esters would be very potent androgens, we investigated the existence of a lipoidal derivative of testosterone (TL) using rats as a model (69). We quantified endogenous TL by RIA after separation and saponification of the nonpolar fraction. We found sizable amounts of TL in fat (2.5 ng/g) and testes (4 ng/g) of male rats and none in the fat of females. There was no TL in any of the other tissues that were examined, including blood and brain. The immunoassayable material released from the TL fraction was confirmed as testosterone by GC/MS.

LE_2, when exogenously administered, is metabolized at an extremely slow rate (70). Since TL is present in much larger concentrations in fat compared with LE_2, the androgen metabolite could be accurately measured. This allowed for the determination of the rate of metabolism of this endogenous family of steroid esters. When the disappearance of TL and testosterone from the fat of male rats after castration was measured (Fig. 6), most of the testosterone disappeared from the fat in 3 h, and none could be detected by 6 h. In contrast,

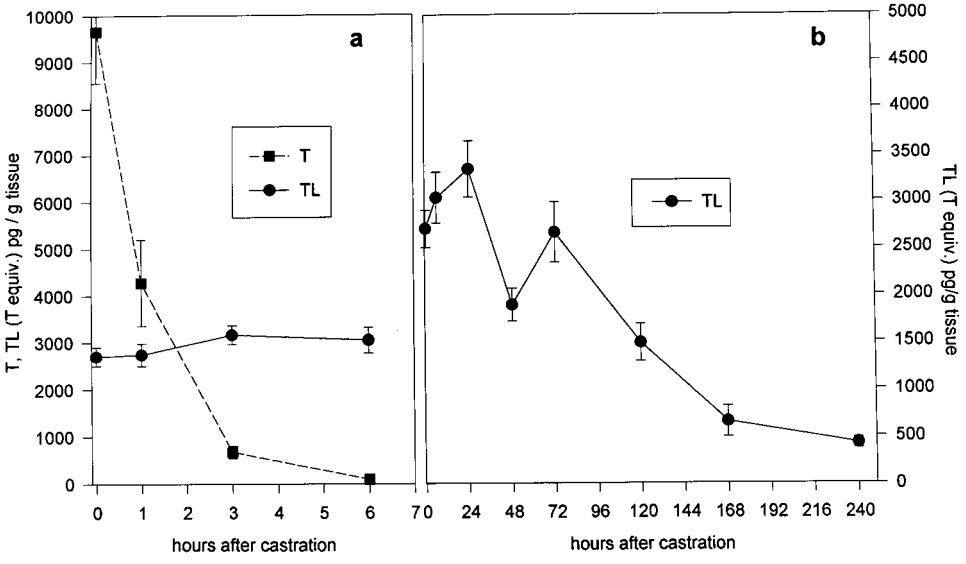

FIG. 6. The effect of castration on the concentration of testosterone esters (TL) in male rat fat. At the indicated time after castration, the animals were killed, the fat was removed, and the TL fraction was purified, saponified, and quantified by RIA for testosterone. In the *left-hand panel*, it is apparent that the unesterified testosterone (T) has disappeared within 3–6 h; during that time there was no detectable change in the TL levels. The *error bars* are SEM. [Reproduced with permission from W. Borg *et al.*: *Proc Natl Acad Sci USA* 92: 1545–1549, 1995 (69) © 1995 National Academy of Sciences, U.S.A.]

3 days after castration, the concentration of TL in fat had decreased only slightly. Even after 1 week, the androgen ester was still present. Thus, the rate of metabolism of TL was exceedingly slow compared with that of "free" testosterone. Indeed, the slow rate of catabolism of the endogenous esters of testosterone was even more pronounced than that originally observed for exogenously administered estradiol esters (70), and it confirms the existence of a protective mechanism that potentiates the hormonal action of the lipoidal derivatives (discussed below).

It is surprising that TL was not found in rat brain because this is the same tissue in which testosterone esterification with fatty acids was first detected (42). It is also of interest because esters of the Δ^5-3β-hydroxysteroids have been found in this tissue (71), another indication of the specificity of the steroid esterification. Although the search for LE_2 in humans was more limited (10) than that for TL in the rat, LE_2 was also found almost exclusively in fat, although it has been shown to be synthesized in a variety of tissues (albeit of different species) (19).

It is not apparent how the sex steroid esters can be localized to fat, as the enzyme system appears to be ubiquitous. While there are several mechanisms that might account for this tissue specificity (different rates of enzymatic hydrolysis and metabolism, different rates of synthesis, different acyltransferases), there is no way to differentiate among these possibilities at present. Since the C-17 esters are such poor substrates for esterase hydrolysis and thus exceedingly long-lived, the first possibility seems to be the least likely. However, the hydrophobic environment within adipocytes may aid in the resistance to esterase enzymes, adding another layer of complexity. The possibility that there might be different enzymes capable of esterifying the 17-hydroxyl group is provocative. In this view, the enzyme in fat could have a higher affinity than the enzyme in other tissues and thus alone would be capable of the esterification of low (physiological) levels of steroids. Multiple isozymes having different K_m values are well known in steroid biochemistry. For example, there are at least two forms of the 11β-dehydrogenase (72, 73) and 5α-reductase (74, 75) enzymes, and the higher affinity enzymes are extremely important in regulating the action of corticoids and androgens, respectively. Nevertheless, the mechanism that results in the differential accumulation of TL and LE_2 is currently not understood.

D. Androsterone esters in breast cyst fluid

Levitz's laboratory showed that androsterone is converted in human breast tumor homogenates to a lipoidal derivative of androsterone, *i.e.*, a nonpolar and saponifiable metabolite (76). This led them to search for this metabolite in human breast cyst fluid since it is known to contain a variety of different steroids and their metabolites (77). They found substantial amounts of the lipoidal derivative of androsterone, averaging approximately 1.4 ng/ml. This amounted to about 17% of the unesterified androsterone in the cyst fluid. The lipoidal derivative was purified in several chromatographic systems, including HPLC, where it migrated with the same retention time as an authentic standard of an androsterone fatty acid ester. The HPLC fractions were analyzed by negative chemical ion mass spectroscopy, which clearly showed the mass ions $(M\text{-}1)^-$ of several different esters as well as their corresponding carboxylate ions. The relative amount of each ester was determined from these spectra by normalizing the mass ions of equimolar solutions of standards. Six different esters were detected; three of them, androsterone oleate (49%), -linoleate (26%), and -palmitoleate (19%), accounted for more than 90% of the total. The source of the androsterone esters was not investigated. While it might be suspected that they are synthesized in the breast cyst fluid by lecithin: cholesterol acyltransferase (LCAT), this is unlikely since 3α-hydroxysteroids such as androsterone are not esterified by this enzyme (78).

IV. Lipoidal Derivatives Synthesized by Lecithin:Cholesterol Acyltransterase

A. Blood

In their studies, Jones and James (79) observed that when several steroids were incubated with blood, they are con-

verted into nonpolar metabolites. They suspected that they were the same metabolites as the lipoidal derivatives that had been found in the adrenal. Indeed, they were saponifiable with alkali. The relative rates of esterification were: pregnenolone 100, dehydroepiandrosterone 60, Δ^5-androstenediol 20, and estradiol 1. Testosterone, 5α-dihydrotestosterone, estrone, androstenedione, and dehydroepiandrosterone sulfate were not esterified. Furthermore, they demonstrated that lipoidal derivatives of the three Δ^5-3β-hydroxysteroids are present in human blood. Of the three, the lipoidal derivative of pregnenolone (PL) is found in the highest concentration, approximately 200 ng/100 ml in both men and women. Dehydroepiandrosterone (DL) is 50 ng/100 ml, and that of Δ^5-androstenediol is 10 ng/100 ml. Thus, the concentrations of endogenous esters are proportional to their rates of synthesis. It was found that blood DL was elevated in subjects with acne and in hirsute women. Roy and Bélanger (80) found that dehydroepiandrosterone was converted into a lipoidal derivative in human serum and that most of the DL was bound to the lipoprotein fraction. As suspected, the esterification was catalyzed by LCAT since it was inhibited by the LCAT inhibitor, dithio-2-bis-nitrobenzoic acid (DTNB). It has been recently shown that dehydroepiandrosterone and pregnenolone are esterified by pure LCAT (81).

It appeared likely that the circulating lipoidal derivatives of steroids in blood were synthesized there by LCAT. Lipoidal derivatives did not seem to be secretory products. It had been previously noted by all investigators that when lipoidal derivatives were synthesized by tissues *in vitro*, they were invariably found only within those cells and not in the incubation media. However, subsequent studies of Bélanger's group opened the possibility that the esters may also be secreted by the adrenal (35). They found that after treatment with ACTH, there was a rapid turnover of these esters in the adrenal concomitant with a marked increase in the esters in blood. While they recognized that the increase of the esters in blood after ACTH administration could have been the result of increased substrate in the blood due to secretion of free Δ^5-3β-hydroxysteroid from the adrenal, secretion of the esters remained a possibility. However, their later experiments in rats and guinea pigs showed that exogenously administered Δ^5-3β-hydroxysteroids were quickly converted into circulating esters, another indication that the esters in blood are not secretory products (82). The lipoidal derivatives formed *in vivo* were present in the lipoprotein fractions, predominantly high density lipoproteins (HDL) and low density lipoproteins (LDL). When the lipoprotein fractions containing the esterified PL and DL were injected into guinea pigs, they had a surprisingly rapid half-life in plasma, about 40 min. Similarly, it was noted that when dehydroepiandrosterone is esterified in human serum, almost all of the DL is in the lipoprotein fractions and almost none is in the residual serum depleted of lipoproteins (80).

When dehydroepiandrosterone is administered to humans by constant infusion, both dehydroepiandrosterone and DL levels increase in blood in such a way that the dehydroepiandrosterone/DL ratio remains constant (81), as would be expected if the DL in blood was formed *in situ* by LCAT. When insulin was administered concurrently with the steroid, the blood levels of both dehydroepiandrosterone and DL decreased. However, the LCAT esterification of dehydroepiandrosterone, but not cholesterol, as measured in blood *in vitro*, increased during insulin treatment. Thus, although dehydroepiandrosterone esterification increased with insulin treatment, the levels of DL decreased, evidence that insulin may increase tissue uptake of DL from blood. Bélanger *et al.* (83) also found that the concentration of DL in blood decreased with aging. The decrease in DL was much less than the age-related fall in dehydroepiandrosterone in blood. Thus, they speculated that although DL decreases with aging, the esterification of dehydroepiandrosterone actually increases.

In separate studies of the interaction of steroids with human lipoproteins, several of the steroids appeared to bind in a nonequilibrium manner (84). It was suspected that this might be an indication of metabolism, esterification by LCAT. DL synthesized by LCAT in the HDL_3 fraction of human plasma was isolated and characterized by single ion monitoring GC/MS (85). Although this technique is very insensitive for steroid esters, nevertheless, it clearly showed the presence of dehydroepiandrosterone linoleate. Further studies (86, 87) showed that of the steroids dialyzed against HDL, six of them, including dehydroepiandrosterone, pregnenolone, Δ^5-androstenediol, estradiol, progesterone, and 5α-dihydrotestosterone, appeared to have an interaction indicative of metabolism. Of these steroids, the first four were converted into lipophilic products; progesterone and 5α-dihydrotestosterone were not. The rate of esterification of the steroids was similar to that previously reported (79). The products of the *in vitro* incubations, PL and DL, were isolated, saponified, and analyzed by gas chromatography. A large number of fatty acids were identified in the saponified fraction. Because the fractions were assumed to be pure (free of other kinds of esters), these results support the hypothesis that the LCAT-synthesized PL and DL are fatty acid esters. As would be expected for products of LCAT, the composition of fatty acids was the same as that of cholesterol esters isolated from the same lipoprotein fraction. Most importantly, the endogenous lipoidal derivatives, PL and DL, present in fresh plasma were isolated from the HDL_3 fraction and analyzed by GC/MS (87). The spectra definitively proved that the lipoidal derivatives in blood were, as suspected, fatty acid esters.

Recently, Bélanger's group investigated the transfer of DL and PL in the lipoprotein fraction of blood (88). They noted that although steroids are esterified by LCAT in the HDL fraction, they accumulate in very low density lipoprotein (VLDL) and LDL as well as the HDL fractions. They presumed that the lipoidal steroids were transferred to the other lipoprotein fractions through the same mechanism that acts on lipoprotein-bound cholesterol esters. The sterol ester is transferred between lipoproteins by the cholesterol ester transfer protein (CETP) (89). However, when they incubated HDL fractions that contained biosynthetic esters of radioactive cholesterol, pregnenolone, or dehydroepiandrosterone with an acceptor VLDL fraction, they found that PL and DL were transferred to VLDL regardless of whether CETP was added. Contrariwise, the addition of CETP was required for the transfer of cholesterol esters from HDL to VLDL.

Furthermore, an antibody to CETP inhibited the transfer of HDL-bound cholesterol esters to other lipoprotein fractions, but it did not affect the transfer of HDL-bound DL and PL. Thus, the transfer of the steroid esters within lipoprotein fractions is different from that of cholesterol esters and does not require the intervention of a transfer protein.

Our laboratory investigated whether LE_2 circulated in human female blood. A nonpolar fraction was isolated from serum extracts, and the E_2 released after saponification was measured by RIA (90). Although we detected LE_2, its concentration could not be accurately quantified because of the high background that was created by the various required manipulations of the extracts. Jones and James (79) did not detect LE_2 in female blood, although they recognized that they did not have the internal standard necessary to assess chromatographic mobility, recovery, etc. They did find, however, that E_2 is a substrate for LCAT, which would be essential for LE_2 synthesis in blood. Subsequently, we reinvestigated this question using the GC/MS technique (10) and confirmed that LE_2 is indeed in blood, albeit in lower amounts than we previously estimated: 2–6 pg/ml in cycling females and 13–25 pg/ml in gonadotropin-stimulated women. The amount of LE_2 in blood is considerably lower than circulating E_2: 2–22% of E_2 in cycling women and about 1% in the gonadotropin-stimulated women.

TL has been reported in the blood of human males (91, 92), but it is likely that some other substance might have cross-reacted in the RIA that was used in those experiments. We have not been able to find TL in human blood after performing a number of experiments (our unpublished results). Bélanger *et al.* could not detect TL in the blood of rats and guinea pigs, even under conditions (androstenedione implants) that produce a dramatic increase in circulating testosterone (82). We also did not detect TL in male rat blood (69). Consequently, it appears that while the estrogen ester, LE_2, circulates in blood, this same metabolite of the androgen, TL, does not. The difference between the two C-17 esters, LE_2 and TL, is consistent with the substrate specificity of the LCAT enzyme, which esterifies steroids in blood. That is, estradiol, but not testosterone, is esterified by this enzyme (79).

Large amounts of corticosteroid acetates, including cortisol acetate, have been reported to circulate in human blood (93, 94). This is rather unexpected given the characterization of the corticoid esters as fatty acid esters, their low rate of esterification (18, 19, 52), and their rapid hydrolysis (95), especially the hydrolysis of steroid acetates (70). There is currently no published confirmation and, as explained in the *Introduction*, there is reason to be concerned about the possibility of an artifactual explanation for those findings.

B. Ovarian follicular fluid

Roy and Bélanger (96) reported that ovarian follicular fluid obtained from subjects treated with gonadotropins contained PL (identified by RIA after saponification). The endogenous PL was isolated and analyzed by GC/MS. While they could not obtain the mass ions of the intact esters (the major signal was M^+ for pregnenolone), this was not unexpected because mass spectra of the intact esters are particularly difficult to acquire by GC/MS analysis. However, they showed that PL migrated on HPLC with retention times identical to several pregnenolone fatty acid esters. Later they found that follicular fluid could catalyze the formation of PL from [^{3}H]pregnenolone and that the enzymatic esterification was suppressed by the LCAT inhibitor, DTNB (97). The biosynthetic ^{3}H-labeled PL was characterized as a family of fatty acid esters (by HPLC analysis) whose composition was identical to that reported for cholesterol esters in serum. Thus, like the steroid esters in blood, PL in follicular fluid is synthesized by LCAT. They showed subsequently that follicular fluid obtained from gonadotropin-stimulated human ovaries contains more than 6 μM PL, over 1000 times greater than the concentration of PL in blood (98). In fact, unlike blood, there is much more PL than free pregnenolone in follicular fluid. Most of the PL in follicular fluid is in the HDL fraction. They also made the interesting observation that charcoal stripping, which adsorbs all of the free steroid from follicular fluid, had no effect on the PL content. This technique is universally used to remove steroids from plasma to produce "steroid free" plasma. It is important to note that the lipoidal derivatives of steroids cannot be removed by this procedure and that significant amounts may be present that could be converted enzymatically into biologically active hormones (see below).

LE_2 is also present in relatively large amounts in human ovarian follicular fluid obtained from women stimulated with gonadotropins (99). The amount of LE_2 in the follicular fluid, approximately 10^{-7} M, was far greater than its concentration in any other tissue and provided a source for the first isolation and characterization of an *endogenous* lipoidal derivative of a biologically active hormone. Five hundred milliliters of ovarian follicular fluid containing 4.4 μg of LE_2 were extracted, and the LE_2 fraction was purified by a combination of chromatographic separations including HPLC. Finally, the LE_2 was resolved by reversed phase HPLC into five different fractions that were identified by mass spectral analysis as estradiol 17-fatty acid esters. The percent composition of the individual esters, as determined both by UV adsorption and RIA (as E_2 after saponification), of the column fractions was: E_2 17-linoleate, 37%; E_2 17-palmitate, 25%; E_2 17-oleate, 18%; E_2 17-arachidonate, 17%; E_2 17-stearate, 4%.

At first it was suspected that the LE_2 was secreted from the ovary rather than synthesized *in situ* by LCAT since E_2 is a poor substrate for LCAT and the concentration of LE_2 is so high in the follicular fluid (almost the same as E_2). Although the fatty acid composition was similar to that reported for the PL in follicular fluid (97) that was synthesized by LCAT, they were dissimilar enough to suggest that PL and LE_2 might have been synthesized by different enzymes. Subsequently, when the comparison was made between the fatty acid composition of endogenous LE_2 in several different follicular fluids and that of LE_2 synthesized *in vitro* by LCAT in the same follicular fluid or in the subjects' serum (100), they were the same. Furthermore, LE_2 synthesized in ovarian cells isolated from the follicular fluid had a completely different composition. Thus, LE_2, like PL in follicular fluid, is synthesized by LCAT. The relatively high concentrations of LE_2 in follicular fluid is probably a result of the combination of the metabolic protection afforded by esterification, the closed environment of the ovarian follicle, and the inability of the

lipoprotein-bound ester to diffuse out of the follicle. The role of LE_2 in the ovarian follicular fluid is not known, but as will be discussed below, LE_2 is a very potent estrogen that could have marked effects on the ovary.

V. Steroid Esterases

Although steroid esters are potent hormones, they do not act directly at the level of the receptor. They require hydrolysis to the parent steroid for their hormonal action. In addition, they are resistant to metabolism. Consequently, their enzymatic hydrolysis by esterase is critical to both their endocrine actions and their catabolism (70). Although esterases that act upon steroid esters are well known, they are for the most part "nonspecific" esterases of leukocytes (101) and other tissues (102). These hydrolytic enzymes have been shown to hydrolyze short-chain esters such as acetate, propionate, etc., of various steroid hormones, including pharmacological steroids. It has been speculated that one of the roles of these enzymes may be the hydrolysis of the naturally occurring steroid esters (103). While this seems a reasonable assumption, there is evidence that the esterase that hydrolyzes estradiol 17-acetate is different from the enzyme that catalyzes the removal of the fatty acid from C-17 of LE_2. Katz *et al.* (13) studied the enzymatic hydrolysis of several C-17 esters of E_2 in the MCF-7 breast cancer cell line. They found that the esterase that hydrolyzes E_2 17-acetate is different from the enzyme that hydrolyzes E_2 valerate and E_2 stearate. Unlike the hydrolysis of the latter E_2 esters, the enzymatic hydrolysis of E_2 acetate is not inhibited by the addition of saturating amounts of the C_5- and C_{16}-esters of E_2. Furthermore, while E_2 treatment of MCF-7 cells stimulates the enzymatic hydrolysis of E_2 acetate, it had no effect on the production of the esterase that acts on E_2 valerate and E_2 stearate. Thus, it appears that the esterase that acts upon the short-chain esters of E_2 (nonspecific esterase) is distinct from the enzyme that hydrolyzes LE_2; *i.e.*, that a more specific enzyme exists. In a separate publication, Katz *et al.* (13) reported that the MCF-7 cell esterase that hydrolyzes E_2 acetate has transacylase activity. While most MCF-7 cells were capable of LE_2 synthesis, one subline, MCF-7 (203P), had no estradiol acyltransferase activity. Incubation of these cells with $[^3H]E_2$ and nonradioactive E_2 17-acetate or E_2 17-valerate (which were added as metabolic traps) led to the formation of $[^3H]E_2$ 17-acetate or $[^3H]E_2$ 17-valerate, respectively. $[^3H]E_2$ esters were not produced when $[^3H]E_2$ alone was incubated with the cells.

Banerjee *et al.* (104) have isolated an esterase from human breast cyst fluid that acts upon estradiol esters. This protein, which has a mol wt of ~90 K and properties of a B type esterase, was shown to cleave several C-17 esters of E_2 including E_2 17-stearate, an ester representative of LE_2. Further analysis of breast cyst fluids from 367 women showed that the esterase activity was present at widely different concentrations (105), and only 39% showed significant activity. Since this enzyme would serve to release free estrogens at specific regions of the breast, the authors speculate that its presence could be a marker for the subset of patients with fibrocystic disease who are at risk for developing breast cancer. Lee *et al.* (106) have compared the esterase activity of bovine placental cotyledons. They have found that throughout purification, the ratio of esterase activity remained constant for estradiol 17-oleate and the lipase substrate, monoacylglycerol. Furthermore, the activity for both substrates decreased in parallel when increasing concentrations of either $HgCl_2$ or phenylmethanesulfonyl fluoride were added as inhibitors. The results indicate that the enzyme responsible for the hydrolysis of LE_2 in this tissue is hormone-sensitive lipase. It is certain that lipase was in the enzyme preparation but, since the enzyme was not pure, the possibility remains that another esterase specific for LE_2 might also have been present. Of course, the likelihood that specific steroid ester hydrolases exist in other tissues was not eliminated.

VI. Lipoidal Derivatives of Steroid Hormones in Insects

Fatty acid esters of the molting hormones, ecdysteroids, are formed enzymatically in various insects. While both polar and nonpolar metabolites of ecdysteroids are formed in insects, in the females of some insects, such as the tick (*Ornithodoros moubata*), the administration of ecdysone or 20-hydroxyecdysone leads solely to nonpolar metabolites that are transferred to their eggs (107). These nonpolar metabolites have been identified in the nymphs of *O. moubata* as fatty acid esters of 20-hydroxyecdysone. Four different C-22 esters of 20-hydroxyecdysone have been isolated from the extract of the entire nymph. They were identified by MS as palmitate, oleate, linoleate, and stearate (108). Palmitate was the major ester (Fig. 7). In another study, the nonpolar metabolites of 20-hydroxyecdysone were isolated from newly laid eggs of the cricket (*Acheta domesticus*). They were characterized by FAB-MS and also found to be C-22 fatty acid esters of the ecdysteroid (109). Seven esters were found in the cricket eggs, including the same four esters previously identified in the tick, plus three additional minor esters, myristate, palmitoleate, and arachidonate. Again, 20-hydroxyecdysone 22-palmitate was the major metabolite. The ovary of the cockroach (*Periplaneta americana*) has been shown to contain an ecdysone acyl-CoA transferase (110). The ecdysone 22-acyltransferase in the midgut of the tobacco budworm (*Helio virescens*) has been characterized and shown to be an acyl-CoA transferase specific for C-22 (111). Although 20-hydroxyecdysone has six hydroxyl groups, three of which are secondary alcohols and thus accessible to esterification, only the C-22 hydroxyl is esterified with fatty acids. Ester-

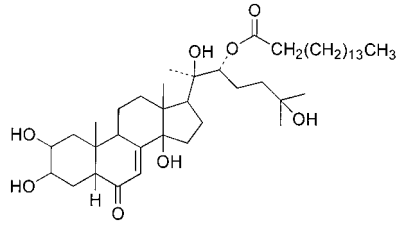

FIG. 7. The structure of the major fatty acid ester of 20-hydroxyecdysone in insects: 20-hydroxyecdysone 22-palmitate [(20R, 22R)-2β,3β,14α,20,25-pentahydroxy-6-oxo-5β-cholest-7-ene-22-yl palmitate].

ification of the C-2 and C-3 hydroxyl group does occur but only with acetate (discussed in Ref. 108). The role of the fatty acid esters of ecdysteroids in insects is not known and may be complex. In several insects, orally administered ecdysteroids are esterified with fatty acids and excreted in the gut and thus may represent deactivated excretory products, although reabsorbtion is thought to be a possibility (112, 113). However, it has been shown in other insects such as the cricket (109) and cockroach (114) that the esters are synthesized in or transferred to the ovary and then to the eggs, where it has been suggested that they represent a storage form of the molting hormone, which supplies free steroid in the developing oocyte during embryogenesis (see references in Ref. 111).

VII. Biological Effects of Steroid Esterification

A. Potent sex steroids

It is widely known that various estradiol esters are therapeutically useful because of their enhanced potency. Most are short-chain esters such as acetate, valerate, caproate, benzoate, and cypionate (cyclopentylpropionate). We tested the effect of C-17 esterification of E_2 with fatty acids in ovariectomized rats, using E_2 17-stearate as a model for LE_2. The ester was injected in aqueous alcohol to eliminate the added complication of the increased solubility of esters in an oil vehicle. There was a large increase in both estrogenic potency and duration regardless of whether the steroid was injected intravenously (115) or subcutaneously (116). However, when the ester was injected subcutaneously to avoid a first pass through the liver, the increase in estrogenicity was especially dramatic (Fig. 8). The increased potency of estradiol fatty acid esters injected in oil has also been reported (117, 118). When compared with other potent estradiol esters such as cypionate, the long-chain ester representative of LE_2 is even more active (119). The same increase in estrogenic potency

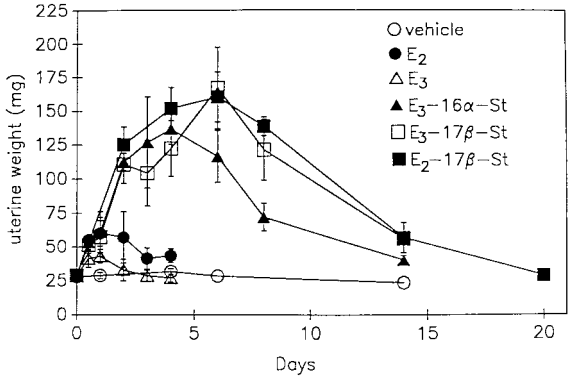

FIG. 8. Estrogenic action of estradiol and estriol fatty acid esters. Equimolar amounts of the steroids dissolved in aqueous ethanol were administered to ovariectomized female mice by a single subcutaneous injection. The animals were killed on the days indicated, and the uteri were removed and weighed. E_2, Estriol; E_3, estriol; E_3 16α-St, estriol 16α-stearate; E_3 17β-St, estriol 17β-stearate; E_2 17β-St, estradiol 17β-stearate. [Reproduced with permission from J. E. Zielinski *et al.*: *J Steroid Biochem Mol Biol* 38:399–405, 1991 (116) with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, U.K.]

was also seen with synthetic LE_3 esters, estriol 16α-stearate and 17β-stearate, injected subcutaneously in aqueous alcohol. The 16α-ester is slightly less potent than the 17β-ester. This would be expected since, unlike the 17β-ester, the 16α-ester is not sterically protected by the 18-methyl group and would be enzymatically hydrolyzed more rapidly than E_3 17β-stearate. It is interesting to note that while E_3 is considered to be a weak estrogen, its low potency due to rapid metabolism can be enhanced by a sustained administration, closer to physiological conditions (120). Apparently, exogenously administered LE_3 can maintain a long lasting E_3 stimulus.

Similar to the estrogen esters, we have found that testosterone fatty acid esters have a greatly increased potency and duration of action when compared with testosterone (our unpublished results). This would be predicted as many alkyl esters of testosterone are used therapeutically for their augmented androgenic action (121). Glucocorticoid fatty acid esters do not bind to the glucocorticoid receptor (95). Unlike the C-17 esters of the androgens and estrogens, they show no increase in potency or duration of hormonal effect when injected in aqueous alcohol (95). This might have been foreseen since corticoid esters are very labile. They are hydrolyzed rapidly by esterases because the C-21 esters are freely rotating primary esters that are activated by the neighboring C-20 ketone, unlike the sterically hindered secondary C-17 esters of estradiol and testosterone. Thus the C-21 esters undoubtedly have a very short biological half-life ($t_{1/2}$).

At the time of the discovery of LE_2, there was some question of whether the estrogen esters could act directly at the level of the estrogen receptor. There had been several studies showing that analogs of short-chain E_2 esters could bind to the estrogen receptor acting as affinity chromatography ligands or covalent affinity ligands that could compete with the binding of radioactive ligands for the receptor. However, it was shown that estradiol esters, including short-chain esters, do not bind to the estrogen receptor except at extremely high concentrations (122). Moreover, when E_2 esters representative of LE_2 are administered *in vivo*, it is E_2 and not the esters that concentrate in the uterine nuclei (115). The activation of the estrogen receptor after the injection of estradiol esters parallels the kinetics of estradiol hydrolysis and accumulation in uterine nuclei (119). Thus, it is clear that the estrogen esters do not act directly at the level of the receptor. It therefore appears likely that the heightened action of LE_2 is the result of an increased resistance to catabolism. We found that fatty acid esterification of E_2 at C-17 increased the $t_{1/2}$ in rats from 2 min for E_2 to more than 6 h for the fatty acid esters of E_2 (70). The nature of the ester was important as E_2 17-stearate had almost a 60% longer $t_{1/2}$ than E_2 17-arachidonate. Metabolic protection of LE_2 was also observed in humans (123). Interestingly, no effect was apparent on the *in vivo* rate of metabolism of short-chain esters of E_2 in rats; E_2 acetate and E_2 hexanoate have about the same $t_{1/2}$ as E_2. Unlike the fatty acid esters comprising LE_2, the short-chain esters are rapidly hydrolyzed (122). Consequently, it seems likely that the increased potency of the pharmacological short-chain E_2-esters is due more to their increased solubility and slow release from the oil vehicle in which they are injected than to an inherent biological mechanism. Contrari-

wise, the biological esterification of the estrogens and androgens with fatty acids potentiates their hormonal actions through their innate resistance to enzymatic hydrolysis; the hydrophobic ester shields the steroid nucleus from catabolic enzymes.

B. Unique actions of the steroid esters in blood

Recently Shwaery *et al.* (124) made the interesting finding of a relationship between LE_2 formation in blood and the antioxidant action of estrogens. Although it is widely recognized that estrogen treatment reduces the age-related increase in coronary artery disease in menopausal women, the mechanism is not known. While it has been observed that estrogens can decrease the *in vitro* oxidation of LDL thought to be implicated in atherosclerosis, the inhibition is accomplished only with supraphysiological (μM) concentrations of estradiol. Consequently, it is generally thought that this direct action is not the mode by which estrogens protect against coronary disease. Shwaery *et al.* found that if male plasma was preincubated with E_2, the LDL fraction subsequently isolated was protected from oxidation. This protection was apparent at 1 nM E_2, an increase in sensitivity of 1000-fold over the micromolar concentrations of E_2 usually required for *in vitro* protection of LDL. When the isolated LDL fraction was analyzed, there was no residual E_2 present, only a nonpolar metabolite that was identified as LE_2. Most importantly, when the esterification of E_2 was blocked with the LCAT inhibitor DTNB, the protective effect was eliminated. Although the mechanism is not yet known, it appears that the esterification of physiological concentrations of E_2 can protect against LDL oxidation. Thus, the formation of LE_2 in blood may be a critical element in a nongenomic effect of estrogens on the prevention of coronary artery disease.

Bélanger's group provided evidence of a possible function for the LCAT-synthesized esters of the Δ^5-3β-hydroxysteroids (98). They hypothesized that circulating lipoprotein-bound lipoidal derivatives of pregnenolone and dehydroepiandrosterone could act as substrates for steroid synthesis and under those circumstances supply hormonal precursors to tissues. It occurred to them that previous reports of ovarian follicular fluid enhancement of progesterone synthesis by granulosa cells might have been caused by PL in the follicular fluid. They made the interesting discovery that the HDL fraction from follicular fluid, but not HDL from plasma, could partially restore progesterone synthesis in porcine granulosa cells in which the cholesterol side-chain cleavage enzyme was inhibited with 10 μM ketoconazole (Fig. 9). Both the HDL from plasma and follicular fluid had the same level of cholesterol esters, but only the HDL from follicular fluid contained large amounts of PL. This experiment opened up the possibility of HDL-bound PL being taken up by the cells and converted into progesterone through the action of an esterase (hydrolysis to pregnenolone), followed by the Δ^5-dehydrogenase-isomerase (oxidation to progesterone). In a more direct study they proved the existence of this pathway. Porcine granulosa cells incubated with HDL or LDL labeled with [^{3}H]PL were shown to synthesize [^{3}H]progesterone. The synthesis of progesterone was stimulated by FSH, implying that uptake or hydrolysis of the lipoprotein-bound

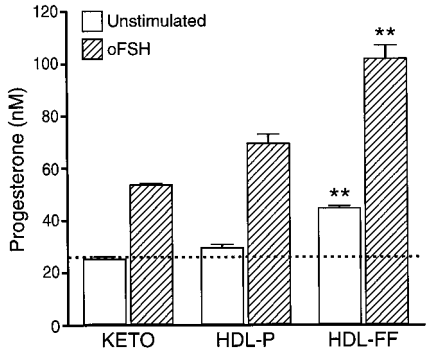

FIG. 9. Stimulation of progesterone secretion of ketoconazole-inhibited granulosa cells by HDL from ovarian follicular fluid. Steroidogenesis in all groups of granulosa cells (porcine) was inhibited with 10 μM ketoconazole. Keto, Control cells incubated without HDL; HDL-P, cells incubated with HDL isolated from human blood; HDL-FF_{DC}, cells incubated with charcoal-treated HDL isolated from gonadotropin-stimulated human ovarian follicular fluid. Charcoal treatment removed most of the free steroids, but not the esters. HDL-P and HDL-FF_{DC} contained the same amount of cholesterol and cholesterol esters, but HDL-P contained 2.5 pmol PL/mg protein while HDL-FF_{DC} contained 513 pmol PL/mg protein. *Error bars* are SEM. **, $P < 0.01$. [Reproduced with permission from R. Roy and A. Belanger: *Endocrinology* 131:1390–1396, 1992 (98). © The Endocrine Society.]

ester is under hormonal control. Chloroquine, an inhibitor of lysosomal degradation, inhibited the formation of unesterified steroids from [^{3}H]PL-LDL. Excess unlabeled HDL or LDL inhibited this reaction with both lipoprotein fractions demonstrating that [^{3}H]PL was taken up into the cells by receptor-mediated internalization.

A similar experiment with guinea pig adrenal cells also demonstrated the synthesis of ^{3}H-labeled steroids when the cells were incubated with [^{3}H]PL-labeled HDL and LDL. ACTH stimulated only LDL-mediated steroidogenesis. The addition of excess unlabeled lipoproteins inhibited synthesis of ^{3}H-labeled steroids with LDL, indicating specific lipoprotein receptor-mediated uptake. Other studies from this group demonstrated that non-steroidogenic tissues could also take up the lipoprotein-bound steroid ester and convert it to the free steroid (125). DL incorporated into human LDL was taken up into ZR-75 human breast cancer cells, where the lipoidal derivative was converted into unesterified dehydroepiandrosterone and Δ^5-androstenediol. Thus, circulating lipoprotein-bound steroid esters can be taken up into cells and converted into free steroids. Apparently steroid esters can act as a reservoir of biologically active hormones that no longer require the cytochrome P_{450} steroidogenic pathway.

VIII. Conclusion

The lipoidal derivatives are a unique class of steroid metabolites that are extremely nonpolar and have properties that are unusual for steroids and their other metabolites. Although they are synthesized in most tissues *in vitro*, their distribution in the body appears to be limited. The fatty acid esters of the sex steroids, TL and LE_2, reside predominantly in fat, while TL is also present in the testes. The mechanism underlying the tissue-specific accumulation is not understood. Nevertheless, it is clear that the enzyme that esterifies the steroids in tissue is a specific enzyme(s). It has been

shown that the acyl-CoA transferase specific for cholesterol, ACAT, does not esterify other steroids, including steroids with the same ring structure as cholesterol such as dehydroepiandrosterone. There are distinct enzymes for the esterification of individual steroids such as E_2 and the corticoids, and possibly a separate enzyme for the Δ^5-3β-hydroxysteroids. Some of the steroid esters, namely those of E_2, pregnenolone, and dehydroepiandrosterone, circulate in blood. The esters are not secreted into blood, but are made *in situ* by the esterification of the free steroids. Interestingly, unlike ester synthesis in tissues, the steroid esters in blood are synthesized by the same enzyme that esterifies cholesterol, LCAT. In a manner that is completely different from all of the other steroids and their metabolites, the esters in blood are bound to lipoproteins. Like the esters synthesized in blood, exogenously added steroid esters also bind to lipoproteins. Even short chain estradiol 17-esters and corticoid-21-esters do not bind to the plasma steroid binding proteins, sex hormone-binding globulin and corticosteroid-binding globulin, respectively (126, 127).

The answer to the important question concerning the role that these esters play is still uncertain, although accumulating evidence indicates some unique actions. The studies of Bélanger have demonstrated a unique pathway by which the steroid esters in blood are taken up by cells through a lipoprotein receptor-mediated mechanism. By contrast, the free steroids are generally thought to enter cells by diffusion. In humans, cholesterol esters bound to LDL are taken up into cells through the LDL receptor. The cholesterol esters synthesized by LCAT are inserted into HDL and are transferred to LDL by CETP. This transfer protein is not required for the movement of steroid esters from HDL to LDL, and it is tempting to speculate that this facilitative transfer provides some insight into the role that these esters play. It has been shown that the Δ^5-3β-hydroxysteroid esters that are taken up into cells are hydrolyzed and converted into active Δ^4-3-ketones, *e.g.*, PL is converted into progesterone (in the adrenal the newly synthesized progesterone has been shown to be converted into corticoids) and DL into testosterone. Nonsteroidogenic cells containing the Δ^5-3β-ol-dehydrogenase-isomerase can also convert these esters into active hormones. In this manner, these circulating steroid esters can act as prohormones, and since they can be "activated" in many tissues, there is a definite possibility of tissue-specific stimulation. The unusual activity of these esters is evident by the report of Shwaery *et al.* that the esterification of estradiol in blood decreases the concentration of E_2 required to inhibit LDL oxidation *in vitro* about 1,000-fold over E_2 by itself (124). The enormous micromolar concentrations of estrogens that are required to inhibit *in vitro* LDL oxidation led most endocrinologists to doubt that E_2 was of physiological significance in that capacity. The finding that the esterification of physiological levels of E_2 produces the active agent, LE_2, could have an enormous impact on the understanding and treatment of arteriosclerosis.

It is clear that the fatty acid esters of the sex steroids, androgens and estrogens, like their short-chain pharmacological analogs, are extremely potent hormones (Fig. 8). Fatty acid esterification is one of the few metabolic transformations that potentiate hormone action. In addition, the esters are prohormones that require hydrolytic enzymes for their activity. Their role in the physiology of the sex steroids is not understood but it is evident from their long-lived action that they may provide target tissue stimulation at times when steroidogenic organs are quiescent and circulating steroid levels are low. Tissue-specific stimulation is possible through the hydrolysis of sex steroid esters (TL and LE_2) and subsequent release of the active hormone from neighboring fat. Paracrine communication by the target tissue could activate specific esterases in adjacent fat, leading to hydrolysis of esters and stimulation only of nearby target organs. Finally, it is prudent to mention that these unusual steroids might have other unforeseen actions. For example, the corticoid esters are unlike other esters of biologically active steroids since they are neither longer lived nor more potent corticoids. The existence of only one specific ester may indicate that it is a specific message with distinct hormonal properties. There is still a great deal about this unusual family of steroids that is not understood, and hopefully more will be revealed about them in the near future.

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Dedicated to Seymour Lieberman on the occasion of his 80th birthday.

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