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Human Steroid Metabolism Revisited

STEROIDAL FATTY ACID ESTERS

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Summary—Several years ago we discovered an unexpected family of steroidal metabolites, steroidal fatty acid esters. We found that fatty acid esters of 5-ene-3β-hydroxysteroids, pregnenolone and dehydroisoandrosterone are present in the adrenal. Subsequently, others have shown the existence of these non-polar 5-ene-3β-hydroxysteroidal esters in blood, brain and ovaries. Currently, almost every family of steroid hormone is known to occur in esterified form. We have studied the esters of the estrogens and glucocorticoids in some detail, and have found that these two steroidal families are esterified by separate enzymes. In a biosynthetic experiment performed simultaneously with estradiol and corticosterone, we established that the fatty acid composition of the steroidal esters is quite different. The corticoid is composed predominantly of one fatty acid, oleate, while the estradiol esters are extremely heterogeneous. Our studies have demonstrated that the estrogens are extremely long-lived hormones, that they are protected by the fatty acid from metabolism. They are extremely potent estrogens, with prolonged activity. Esterification appears to be the only form of metabolism that does not deactivate the biological effects of estradiol. We have demonstrated the biosynthesis of fatty acid esters of estriol, monoesters at both C-16α and C-17β. They too are very potent estrogens. These fatty acid esters of the estrogens are the endogenous analogs of estrogen esters, like benzoate, cypionate, etc., which have been used for decades, pharmacologically because of their prolonged therapeutic potency. We have found that the estradiol esters are located predominantly in hydrophobic tissues, such as fat. Sequestered in these tissues, they are an obvious reservoir of estrogenic reserve, requiring only an esterase for activation. To the contrary the biological activity of the fatty acid esters of the glucocorticoid, corticosterone, is not different from that of its free parent steroid. We have shown that the rapid kinetics of its induction of gluconeogenic responses is caused by its labile C-21 ester which is rapidly hydrolyzed by esterase enzymes. While it appears that the physiological role of the estrogen esters may be related to their long-lived hormonal activity, the role of the other families of steroidal esters is not yet apparent. They, and perhaps the estrogen esters as well, must serve other purposes. Indeed they may serve important biological functions beyond those which we ordinarily associate with steroid hormones.

For over half a century alkyl esters of steroid hormones have been used as pharmacological agents because they are excellent drugs, with extremely potent and long lasting actions [1]. Despite the common usage of the synthetic steroidal esters, it was not suspected that similar non-polar esters of steroids might exist naturally, e.g. that the pharmacologic esters are synthetic analogs of endogenous metabolites. While it is well recognized that steroid hormones are conjugated, those metabolites are very polar and usually ionic, such as glucuronides and sulfates. The possibility that steroidal fatty acid esters, at least 5-ene-3β-hydroxysteroid esters, might exist was evinced in experiments that we had undertaken to study the substrate specificity of the cholesterol side chain cleavage enzyme, the C-20,22 desmolase. This enzyme which is extremely substrate specific, and tolerates little structural modification of cholesterol, inexplicitly converts cholesterol sulfate to pregnenolone sulfate at a very high rate [2, 3]. C-20,22 cleavage of the sterol sulfate is the beginning of the "steroid sulfate pathway" which leads to dehydroisoandrosterone sulfate [4–6]. The ability of the cholesterol sidechain cleavage enzyme to utilize these two remarkably different sterols, cholesterol and cholesterol sulfate, as substrates, led us to examine the effect of structural modifications at C-3 on enzyme specificity [7]. As we suspected, various polar additions at the 3β-hydroxyl (glucuronide, phosphate, etc) led

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to compounds that were very poor substrates. Only cholesterol sulfate could serve efficiently which demonstrated the unique role of the steroid sulfates in steroidogenic pathways. However, the conversion of another cholesterol derivative was even more surprising. We found that cholesterol acetate is an excellent substrate, equal to cholesterol itself. The conversion of cholesterol acetate to pregnenolone acetate demonstrated the possibility that alkyl esters of steroids might exist.

LIPOIDAL DERIVATIVE OF 5-ENE-3β-HYDROXYSTEROIDS IN THE ADRENAL

We had presumed that cholesterol acetate would not be C-20,22 cleaved because it was generally believed that cholesterol fatty acid esters are not substrates for the side chain cleavage enzyme. We confirmed that when the alkyl group of the ester is lengthened the rate of C-20,22 cleavage declines until fatty acid esters are not converted to C_{21}-steroidal esters at all [7]. However, the adrenal has a large variety of diverse cholesterol esters and we realized that the conversion of cholesterol acetate into pregnenolone acetate might be an indication that one or another could be a steroidogenic substrate which would lead to a C_{21}-steroidal ester. Since it was not clear what the structure of the ester would be, we decided to search for a non-polar derivative of pregnenolone in the adrenal. We isolated a non-polar lipid fraction from bovine adrenals, in which there was neither pregnenolone nor any polar conjugate. When this fraction was hydrolyzed pregnenolone was produced [8]. This critical experiment demonstrated that the adrenal gland contains a non-polar, saponifiable pregnenolone metabolite. We named this compound the lipoidal derivative of pregnenolone, PL, to convey its hydrophobic character and uncertain structure. Although the lipoidal derivative of pregnenolone and many of the other steroid hormones have been identified as fatty acid esters we will continue to use the term lipoidal derivative in describing these unusual metabolites.

There are two sources for PL. The first, through C-20,22 cleavage, is the pathway we had originally postulated. However, there is an additional possibility, the formation of PL though direct metabolism of pregnenolone. We asked whether pregnenolone could be converted into PL in the adrenal by incubating pregnenolone with adrenal preparations. We found that both pregnenolone and dehydroisoandrosterone are converted into non-polar metabolites PL and DL, respectively, and that each of these metabolites could be saponified to the free steroid [9]. We also found in these studies that PL is heterogeneous and could be chromatographically separated into at least three fractions. Subsequently, PL biosynthetically produced from pregnenolone was isolated and identified as a mixture of fatty acid esters [10]. The question still remained as to whether there is a biosynthetic pathway in which a lipoidal derivative of cholesterol is converted into PL. There is indirect evidence for this pathway or at least one that is different from that which converts pregnenolone to PL. We found that there are several types of PL in the adrenal. We added [^{3}H]PL that had been made biosynthetically to a lipid extract from bovine adrenals and chromatographed the mixture on a column of florisil [11]. Three fractions were isolated which contained endogenous PL, but the biosynthesized [^{3}H]PL was present in only one. Thus the other two fractions of PL are not synthesized from pregnenolone. The obvious source would be from cholesterol metabolites. The resolving power of the florisil column is poor and it is probably insufficient to separate compounds of similar structure. So it may be inferred that the difference between the 3 types of PL are not small. While PL biosynthesized from pregnenolone has been characterized, the other 2 fractions have not yet been identified. Subsequently it has been demonstrated that there are biosynthetic pathways in which lipoidal derivatives take part. [^{3}H]PL, made by the incubation of [^{3}H]pregnenolone with adrenals, is converted by adrenal subcellular organelles into [^{3}H]17α-OH-PL and [^{3}H]DL [12]. While the yields were low, nevertheless, the 17α-hydroxylase and the 17,20-desmolase enzymes can utilize these non-polar metabolites as substrates.

PL AND DL IN BRAIN, OVARIES AND BLOOD

While PL and DL are synthesized and are present endogenously in the adrenal gland, these unusual metabolites have been found in different tissues. Using gas chromatography/mass spectrometry, fatty acid esters of pregnenolone and allopregnanolone (3β-hydroxy-5α-pregnan-20-one) have been identified in bovine corpora lutea [13]. In addition, it has been shown that human follicular fluid obtained from patients stimulated with gonadotrophins contains relatively large amounts of PL [14]. The concentra-

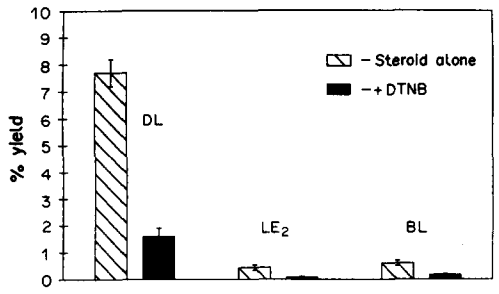

Fig. 1. Esterification of steroids in rat serum. Diluted rat serum was incubated with [^{3}H]dehydroisoandrosterone, [^{3}H]E_2 or [^{3}H]corticosterone and the lipoidal derivatives of dehydroisoandrosterone (DL), E_2 (LE_2) and corticosterone (BL) were isolated by HPLC and quantified. DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid).

tion of PL in follicular fluid is 2 fold large than that of free pregnenolone, and it consists mainly of unsaturated fatty acids. A highly provocative finding is that pregnenolone, dehydroisoandrosterone and a saponifiable form of both steroids, presumably PL and DL, is present in the brain [15, 16]. While the role of the 5-ene-3β-hydroxysteroids and their fatty acid esters in the brain has not yet been clarified, nevertheless the fact that they are concentrated there would appear to indicate that they have a function, and so they have been termed neurosteroids. The esterification of 5-ene-3β-hydroxysteroids also been demonstrated in breast cancer cells in culture [17, 18]. DL and PL have been shown to circulate in human blood [19]. In fact it has been shown that dehydroisoandrosterone is esterified in blood by lecithin:cholesterol acyltransferase LCAT, [20, 21] and to fractionate with the lipoproteins. Our studies have compared the esterification of dehydroisoandrosterone with estradiol (E_2) and corticosterone in rat blood. As can be seen in Fig. 1, dehydroisoandrosterone is efficiently esterified to DL, and this transformation is blocked by the LCAT inhibitor 5,5′-dithiobis-(2-nitrobenzoic acid). While both E_2 and corticosterone are esterified, the rate of esterification of both the C_{18} and C_{21} steroid is much less than that of dehydroisoandrosterone. It has been noted [20] that the association of DL with lipoproteins may imply that steroid esters can enter cells by receptor mediated pathways of lipoprotein internalization. Thus, PL and DL may have physiological roles quite different from that of their unesterified parent steroids.

LIPOIDAL DERIVATIVES OF ESTROGENS

Shortly after we had discovered that lipoidal derivatives of 5-ene-3β-hydroxysteroids existed, we recognized that similar metabolites of biologically active steroids could have a considerable impact on their hormonal activity. It is well known that alkyl and aromatic esters of E_2 are very powerful estrogens, and so we undertook studies to determine if E_2 was metabolized into an endogenous form of these drugs, a lipoidal derivative, LE_2 [22]. Indeed, as we suspected several tissues converted E_2 into a non-polar saponifiable metabolite. In a large scale experiment with bovine endometrium, we isolated LE_2 and characterized it as a complex mixture of C-17 fatty acid esters of E_2 (Fig. 2) [23]. The phenolic C-3 esters are not synthesized. In these experiments we showed that there was appar-

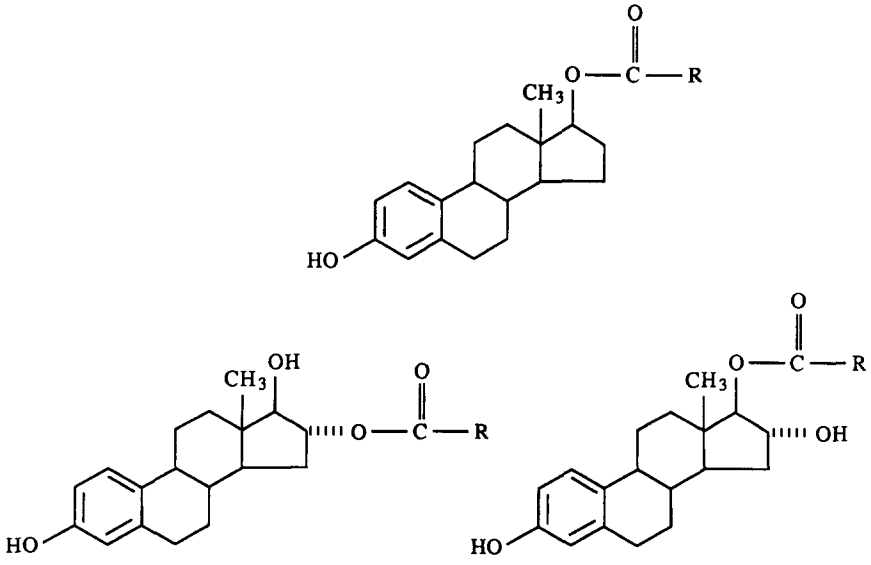

Fig. 2. Lipoidal derivative of estrogens. Top: LE_2, C-17 esters of E_2; bottom left: 16α-LE_3, C-16 esters of E_3; bottom right: 17β-LE_3, C-17-esters of E_3. R—C=O represents any fatty acid.

ently some specificity in the biosynthesis of LE_2, for the fatty acid composition is very different from most of the other lipids within this tissue. Subsequently several investigators have confirmed the synthesis of LE_2 in various tissues [24–29] and an acyl CoA transferase that catalyzes the esterification of E_2 has been characterized in microsomes from bovine placenta [30, 31] and liver [32]. The synthesis of LE_2 in estrogen target tumors such as breast cancer has been recognized as potentially important in the maintenance of the tumor [25]. In addition to E_2, estriol (E_3), the major estrogen of human pregnancy, has been shown to be converted into a lipoidal derivative, LE_3 [33] in a human breast cancer cell line, ZR-75. Although in those studies LE_3 was not characterized. We have recently isolated and identified LE_3 produced by rat lung, a tissue with a relatively high capacity to produce LE_2. The extract of rat lung incubated with E_3 was purified in a variety of chromatographic systems [34]. In one of these HPLC systems (silica gel) we separated LE_3 into 2 peaks, which we identified as E_3-16α-fatty acid esters and E_3-17β-fatty acid esters (Fig. 2). Consequently, both D-ring positions in E_3 are esterified. The fatty acid composition of both are identical and similar to that of LE_2 made in the same tissue [35]. It is not yet known whether the same enzyme esterifies both 16α and 17β of E_3 as well as 17β of E_2. However, as described below all 3 of these esters are very powerful hormones or prohormones.

CORTICOSTERONE ESTERS, BL

Pearlman *et al.* [36] found that when corticosterone is incubated with rat mammary tissue, a non-polar metabolite of corticosterone is formed which they called the acyl derivative of corticosterone, and which we will take the liberty of renaming the lipoidal derivative of corticosterone, BL. It was shown that BL was acylated at C-21. Subsequently, BL was isolated and identified as the 21-fatty acid ester of corticosterone [37] (Fig. 3). Interestingly, the fatty acid composition of BL is very different than that of the heterogeneous estrogen esters, LE_2 and LE_3. While BL is also heterogeneous, several fatty acid esters were identified, most were minor components. There is one major component of BL, corticosterone-21-oleate. Likewise, the mineralocorticoid, aldosterone, is converted into a lipoidal derivative by mammary tissue, and it consists mainly of aldosterone-oleate [38]. The lipoidal derivatives of aldosterone and corticosterone were both isolated from rat mammary tissue, a tissue in which LE_2 synthesis had not been studied. In order to compare BL and LE_2 synthesis in the same tissue, we isolated both steroidal esters from parallel incubation of E_2 and corticosterone with rat mammary tissue [35]. As reported by Pearlman *et al.* [37] corticosterone was converted to a predominant C-21 fatty acid ester, oleate, which comprises about 80% of the total BL. Small amounts of linoleate, stearate and arachidonate were also found. To the contrary LE_2 was very heterogeneous and contained many fatty acids not present in BL, including the medium chain acids C_8, C_{10} and C_{12}. We also found that the relative rate of formation of BL and LE_2 in different tissues varied greatly, which indicated that the rate of synthesis of each steroid differed independently. If the same enzyme esterified E_2 and corticosterone it would be assumed that the relative synthesis of both esters would be constant from tissue to tissue and also that their composition should be the same. Since neither occur, it suggests that the enzyme that esterifies C-21 of corticosterone is different than the one that esterifies C-17 of E_2. The existence of different enzyme systems for the two classes of steroid hormones opens the possibility that separate physiological mechanisms and controls govern their synthesis.

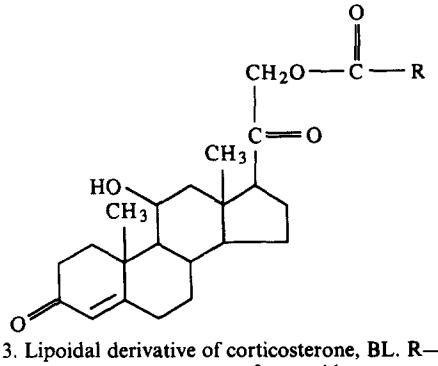

Fig. 3. Lipoidal derivative of corticosterone, BL. R—C=O represents any fatty acid.

LIPOIDAL DERIVATIVE OF ANDROGENS

As mentioned above one of the first experiments which showed that steroids hormones are esterified, demonstrated the formation of testosterone fatty acid esters in rat brain [39]. Even before that it had been reported that testosterone is acetylated [40]. In reality, however, it is likely that the metabolite isolated in that study was not an acetate but a fatty acid

Table 1. Endogenous steroidal fatty acid esters

Steroid	Tissue source	Method of identification	Ref.
Pregnenolone	Bovine adrenal	DID	[8]
17α-OH-Pregnenolone, DHIA	Bovine adrenal	DID, RIA	[11]
Pregnenolone, allopregnanlone	Bovine corpus luteum	GC/MS	[13]
Pregnenolone, DHIA	Human blood	RIA	[19]
Pregnenolone	Human follicular fluid	MS, RIA	[14]
Pregnenolone	Rat brain	RIA, MS	[15]
DHIA	Rat brain	RIA	[16]
Estradiol	Human blood	HPLC-RIA	[52]
Estradiol	Human blood	DID	[53]
Androsterone	Human breast cyst	MS	[43]
Testosterone	Human blood	RIA	[41]
20-Hydroxy-ecdysone	Ticks	MS	[54]
Ecdysone	Cricket eggs	MS	[55]

MS = mass spectroscopy; DID = double isotope dilution; HPLC = high performance liquid chromatography GC = gas chromatography; and RIA = radioimmunoassay.

ester; the chromatographic systems available at that time were not capable of resolving the C_2 ester from longer chain fatty acid esters. More recently, testosterone and the 5α-dihydrotestosterone have been shown to be esterified by a human breast tumor cell line [33]. Of potential physiological and pathological importance is the recent discovery that sizeable amounts of testosterone fatty acid esters circulate in human male blood [41]. If the testosterone esters are as potent as androgens as the E_2 and E_3 esters are as estrogens, (see below), they may provide a substantial androgenic signal which has been previously unrecognized. One of the major metabolites of testosterone, androsterone, is esterified by human breast tumors [42]. Comparatively large amounts of androsterone fatty acid esters have been identified in human breast cyst fluid [43]. With few exceptions, most families of steroid hormones have been shown to be esterified and many have been identified endogenously in tissues (see Table 1).

BIOLOGICAL ACTIVITY, LE_2 AND LE_3

While the physiological significance of the lipoidal derivative of all families of steroid hormones is not certain, studies of the biological activity of the estrogen esters were undertaken in order to attempt to illuminate their role. Certainly one would have predicted from the pharmacological analogs that the esters comprising LE_2 would be very active estrogens. In a series of studies with a representative ester we showed that E_2-17-stearate is an extremely potent estrogen which produces a sustained estrogenic response [44, 45]. In immature rodents [44] the ester activates the hypothalamic–pituitary–ovarian axis and advances puberty, a response that can only be mimicked by multiple injections of E_2. With LE_3, the estrogenic response is even more dramatic, for through esterification a very weak estrogen, E_3, evolves into an extremely potent one, LE_3 [46]. The uterotrophic stimulation resulting from a single subcutaneous injection of E_2, E_3, E_2-17β-stearate, E_3-16α-stearate and E_3-17β-stearate is shown in Fig. 4. It can

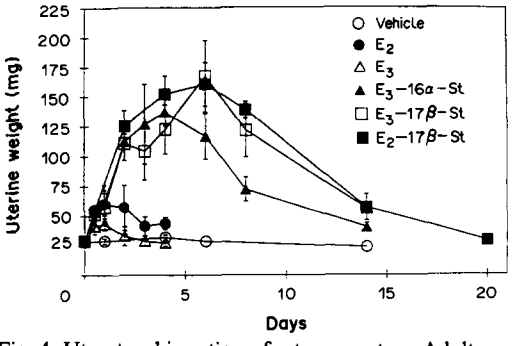

Fig. 4. Uterotrophic action of estrogen esters. Adult ovariectomized female mice were injected subcutaneously with either 132 nmol/k of E_2 or its ester, or 264 nmol/k of E_3 or its esters. The steroids were dissolved in 200 μl of aqueous alcohol. At the indicated times, the animals were killed, uteri were removed, dissected and weighed. E_2-17β-St = E_2-17-stearate; E_3-16α-St = E_3-16-stearate; E_3-17β-St = E_3-17-stearate. (The structure of the 3 steroidal esters are depicted in Fig. 2 as LE_2, 16α-LE_3 and 17β-LE_3, respectively.) Error bars are SEM.

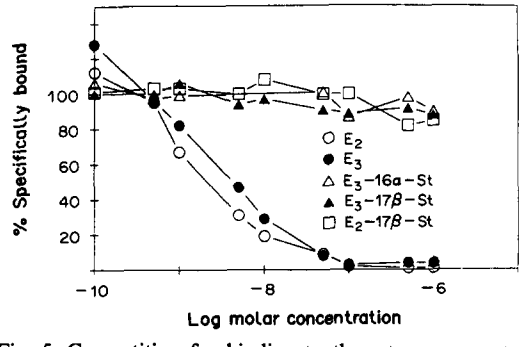

Fig. 5. Competition for binding to the estrogen receptor. The indicated concentrations of steroids were incubated overnight at 0°C with [^{3}H]E_2 and rabbit uterine cytosol. Abbreviations are defined in the legend to Fig. 4.

be seen that the esters comprising LE_2 and LE_3 produce a strong and sustained estrogenic stimulation compared to the free steroids. In fact a single injection of the estrogen esters results in a hormonal signal that lasts about 2 weeks.

We asked the question of how the E_2 and E_3 esters induced their estrogenic response and produced a protracted stimulation. There was reason to believe that LE_2 and LE_3 could act directly as estrogens, without further metabolism. D-ring esters of E_2 have been reported to bind to the estrogen receptor, and furthermore both monoesters of E_3 have free hydroxyl groups in their D-ring, a requirement for binding to the receptor. However, neither LE_2 [47] nor either of the esters of LE_3 [46] compete for binding of $[^3H]E_2$ to the estrogen receptor in uterine cytosol (Fig. 5). Likewise, *in vivo* when $[^3H]E_2$-17-stearate is injected into ovariectomized rats, only $[^3H]E_2$ accumulates in uterine nuclei [44]. When compared to the administration of $[^3H]E_2$, there is a delay in nuclear radioactivity with $[^3H]E_2$-17-stearate, and this delay corresponds exactly to the induction of nuclear estrogen receptors with E_2-17-stearate [45]. All of these results strongly indicate that LE_2 and LE_3 do not act directly but require enzymatic hydrolysis to E_2 and E_3, respectively for their action.

In order to determine how LE_2 produces the prolonged estrogenic stimulation, we synthesized a series of esters of $[^3H]E_2$ and studied their rate of metabolism in rats [48] and humans [49]. The short chain esters, up to C_6 were metabolized very rapidly, at about the same rate as E_2. In contrast, as the chain length of the esters was increased metabolism decreased, and at the dimensions of the naturally occurring esters of LE_2, such as C_{18}, metabolism was extremely slow. Interestingly, in the rat experiments [48] the blood clearance rate of the esters was also measured and a different pattern was found. Both clearance and metabolism were similar for the esters up to C_{12}, both rates decreased as the chain length increased. In contrast, at C_{14} through C_{18}, while these esters have exceedingly slow metabolism, their rates of clearance increased progressively. Thus as the esters approached the size of the natural esters of LE_2, blood clearance increases. Perhaps an active process regulates the uptake of the esters from blood. Nevertheless, it is very clear from these experiments that fatty acid esterification of the D-ring of steroidal estrogens dramatically protects the steroid nucleus from metabolism and prolongs their biological life. It is this protection from metabolic deactivation that causes a long-lived hormonal stimulation.

BIOLOGICAL ACTIVITY OF CORTICOSTERONE ESTERS, BL

It seemed apparent from the experiments on the estrogen esters LE_2 and LE_3 that the biological consequence of steroid esterification is a protected, extremely potent hormone. To show that this was true for the esters of all families of steroid hormones, we compared the effect of an ester of corticosterone, corticosterone-21-stearate, with corticosterone, on the induction of hepatic tyrosine aminotransferase (TAT) in adrenalectomized rats [50]. Unexpectedly, there is no difference between the ester and the unesterified corticoid, in both the magnitude or the duration of the induction of this enzyme (Fig. 6). While there are many reasons that might explain the difference in biological activity between the 2 families of steroid esters, corticoids and estrogens, the most obvious include interactions with the respective hormone receptors or in their rates of metabolism. While the E_2 and E_3 esters do not bind to the estrogen receptor, it was possible that because the oxygen functions at C-11 and C-20 were free in corticosterone esters, that esterification at C-21 would not interfere with binding to the glucocorticoid receptor. The consequence would be that the esterified steroid could act directly with the same potency and kinetics as corticosterone itself. However, when we examined the glucocorticoid receptor binding of esters representative of BL, corticosterone oleate and stearate, both competed for the binding of [^{3}H]dexamethasone to the glucocorticoid

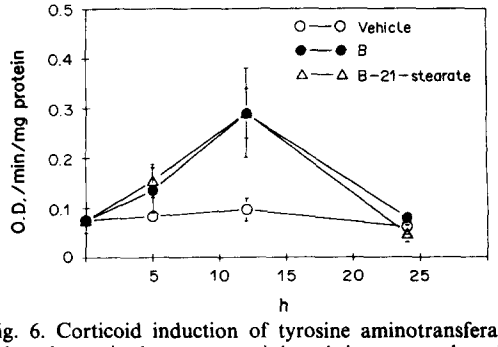

Fig. 6. Corticoid induction of tyrosine aminotransferase. Adrenalectomized rats were injected intravenously with 0.32 μmol/100 g body wt of either B, corticosterone, or B-21-stearate, corticosterone-21-stearate. At the indicated times animals were killed and hepatic tyrosine aminotransferase determined. Error bars are SD.

receptor in rat liver cytosol only poorly compared to corticosterone [50]. It is likely that even the limited competition is not caused by the esters themselves, rather by their conversion to corticosterone through esterase action. Thus like the E_2 esters, the corticosterone esters are probably not ligands for their receptor.

From the results of the receptor studies it is doubtful that the esters of BL could act directly *in vivo* without prior cleavage of the fatty acid. It appeared likely that the difference between estrogen and corticoid esters would reside in their metabolism. Consequently, we measured the rate of hydrolysis of the esters of both families of steroids in rat liver, comparing corticosterone-21-stearate to E_2-17-stearate [50]. As predicted, corticosterone-21-stearate is hydrolyzed at a much greater rate, 10 to 25 fold, than E_2-17-stearate. The difference in the hydrolysis of the 2 esters can be easily explained. The 17β-ester of E_2-17-stearate is a secondary ester, sterically hindered by the bulky C-18 methyl group, making it relatively inaccessible to an esterase enzyme. It has been shown that an esterase that readily hydrolyzes short-chain C-17 esters of E_2, can only poorly hydrolyze E_2-17-stearate [47, 51]. Contrariwise, the C-21 ester, corticosterone-21-stearate, is a primary ester, freely rotating, enzymatically accessible, and in addition it is activated by the C-20 ketone. The combination of these factors make corticosterone-stearate an excellent substrate for hydrolytic enzymes. Consequently, it is highly likely that the difference in the kinetics of TAT induction by corticosterone-stearate compared to the kinetics of estrogenic stimulation by estradiol-17-stearate can be explained by the large differences in their rate of enzymatic hydrolysis. Since the esterification of the corticoids appears not to be related to an increased biological half life, the question remains as to the physiological role that they might play. In addition the role of the lipoidal derivatives of the 5-ene-3β-hydroxysteroids is still unknown. Contrariwise, it appears that the lipoidal derivatives of the estrogens serve the function of preformed hormonal signals which are sequestered in hydrophobic tissues. Although we have shown that LE_2 circulates in human female blood [52, 53], more recently we have found that this E_2 ester is concentrated in relatively large amounts in fat (unpublished observations). This senescent hormone, requires only the action of an esterase for activation and not *de novo* steroidogenesis. The local production of E_2 through hydrolytic action in these tissues can lead directly to the stimulation of neighboring estrogen target tissues. In addition, there is an unmistakable possibility that all of the steroidal esters have physiological roles that have not yet been delineated.

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