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Long-lived testosterone esters in the rat

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ABSTRACT **Over the past decade it has become increasingly clear that steroid hormones are enzymatically esterified with fatty acids. These steroidal esters are the natural analogs of synthetic esters that are used therapeutically. One such family of pharmacological steroids is the synthetic alkyl esters of testosterone, androgens with great hormonal potency. We have investigated whether testosterone esters exist naturally by using the rat as a model. Most tissues of male rats, including blood, have very little if any ester (quantified by immunoassay as a nonpolar saponifiable metabolite), but fat and testes have sizable quantities, ≈3 ng of testosterone equivalents per g of tissue. Testosterone in fat averages 9 ng/g. The fat from female rats and long-term (>2 weeks) castrated males has no detectable testosterone ester. The presence of testosterone esters was confirmed by GC/MS, which clearly showed the presence of testosterone in the hydrolyzed ester fraction of fat from intact males but not long-term castrates. Upon castration, testosterone levels in the fat completely disappear within 6 hr. To the contrary, it is not until 48 hr after castration that a measurable fall in the testosterone ester fraction was observed; even after 10 days a small amount of ester is still present in the fat. These experiments demonstrate the existence of a previously unknown androgen with a potentially important physiological impact; testosterone esters, natural analogs of potent therapeutic agents, occur in the fat where they can serve as a reservoir of preformed androgen to stimulate neighboring target tissues.**

Although fatty acid esters of sterols, such as cholesterol, have been known for decades, the existence of naturally occurring fatty acid esters of steroids is a much more recent discovery. In 1979, putative steroidal esters of the Δ^5-3β-hydroxysteroids, pregnenolone (1), dehydroisoandrosterone, and 17α-hydroxypregnenolone (2), were discovered in the adrenal gland. They were named lipoidal derivatives to convey their nonpolar nature and their ability to be converted back to the parent steroid as a result of mild hydrolytic procedures. They were subsequently identified as fatty acid esters (3). These findings raised the possibility of the existence of similar esters of biologically active steroids. Such compounds would be the natural analogs of synthetic steroidal esters that have been used pharmacologically as extremely potent and long-lived hormones. The well known therapeutic use of one such family of pharmacological steroid hormone esters, the estrogens (4), led to experiments which showed that estradiol (E_2) is biosynthetically converted into a lipoidal derivative, LE_2 (5), a nonpolar metabolite, which was identified as a family of C-17 fatty acid esters of E_2 (6). Although LE_2 is not estrogenic when esterified (7)—i.e., it does not bind to the estrogen receptor directly (8)—it is converted to E_2 by esterase action. The fatty acid esters comprising LE_2 are extremely long-lived (9) and, thus, act as a reservoir of E_2. They represent the most potent of the naturally occurring steroidal estrogens (10, 11).

There are now numerous studies showing that fatty acid esters of almost all of the families of steroid hormones are synthesized *in vitro* (12, 13). However, there are only a few which demonstrate that steroid esters exist endogenously. After the esters of the Δ^5-3β-hydroxysteroids were discovered in the adrenal gland (1, 2), esters of dehydroisoandrosterone and pregnenolone were found in blood (14) and pregnenolone esters were found in human ovarian follicular fluid (15). Fatty acid esters of reduced metabolites of progesterone and testosterone have been isolated and characterized by MS, revealing androsterone esters in human breast cyst fluid (16) and allopregnanolone (as well as pregnenolone) in bovine corpora luteum (17). There are very few studies of endogenous esters of biologically active steroids. Esters of E_2 are present in limited concentration in blood (17), with much greater amounts in fat (18). Relatively large amounts of E_2 fatty acid esters have been found in human ovarian follicular fluid, enabling their complete characterization (18). Other reports of esters of active steroids are more tenuous. Esters of testosterone (19, 20) and the corticoids, cortisol and corticosterone (21, 22), have been reported to circulate in sizable amounts in human blood. Although the report of corticoid esters in blood was first published in 1960, there has been no independent confirmation. The evidence for testosterone esters in blood is uncertain since neither long-term inhibition of steroidogenesis nor castration reduced the concentration of the putative compound (20).

The existence of natural esters of androgens is of importance because, like estrogen esters, synthetic alkyl esters of androgens have been used therapeutically for decades due to their high potency and prolonged action (23). Thus, biological esterification of androgens, such as testosterone, would be expected to have a dramatic effect on both the potency and the life of the male hormone. This paper reports a study in which the existence of fatty acid esters of testosterone (TL) in tissues of the male rat was examined and the hypothesis was tested that TL is long-lived when compared to testosterone.

MATERIALS AND METHODS

[1,2,6,7,16,17-^{3}H]Testosterone (100 Ci/mmol; 1 Ci = 37 GBq) purchased from New England Nuclear was refluxed with alkali to remove labile ^{3}H (24) and then purified by HPLC (25); final specific activity was 92 Ci/mmol. Testosterone stearate and [^{3}H]testosterone stearate were synthesized by esterification with stearyl chloride and purified exactly as described (25).

Sprague–Dawley rats (Charles River Breeding Laboratories) between 3 and 5 months old were castrated under methoxyflurane (Metofane; Pitman–Moore, Washington Crossing, NJ) anesthesia (day 0). At the stated times, animals were decapitated while under Metofane anesthesia and the tissues were removed and immediately frozen. Blood was obtained by cardiac puncture, placed on ice, and centrifuged at 4°C, and the serum was removed. Fat from various areas was combinded and mixed. Ap-

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Abbreviations: TL, testosterone ester; E_2, estradiol; LE_2, E_2 lipoidal derivative; THF, tetrahydrofuran.
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proximately 250 mg of each tissue was weighed, transferred to a test tube (16 × 125 mm) containing 2 ml of methanol and homogenized with two 10-sec bursts of a Polytron homogenizer (Brinkmann). To correct for experimental losses, a representative fatty acid ester, 5000 cpm of [^{3}H]testosterone stearate (13 pg; testosterone molar equivalent), was added as an internal standard in 50 μl of ethanol. When testosterone was measured, an internal standard of [^{3}H]testosterone, 5000 cpm, was also added. The suspension was mixed, 4 ml of chloroform was added, and it was Vortex mixed again. Two milliliters of water was added and, after thorough mixing, the suspension was clarified by centrifugation at 1600 × *g* and the bottom organic layer was removed with a Pasteur pipette. The aqueous layer with the tissue residue was extracted again with 4 ml of the organic layer obtained by partitioning chloroform/methanol/water in the ratio 2:1:1. The organic extracts were combined, evaporated under N_2, and then put under vacuum at 50°C for 10 min to remove all traces of alcohol.

With serum, a slightly different extraction procedure was used to increase the extraction yield. Four milliliters of freshly distilled tetrahydrofuran (THF) was added to 1 ml of serum, followed by the ^{3}H internal standards and then 1 ml of brine. The solution was vigorously mixed and then centrifuged. The THF was removed and the aqueous residue was extracted again with an additional 2 ml of THF. The THF layers were combined and evaporated under N_2. The residues from the extracts of the various tissues and serum were dissolved in 1 ml of benzene/hexane (3:1) and transferred to a column (6 × 0.5 cm) of alumina (3% H_2O) equilibrated in the same solvent. The column was washed with 10 ml of benzene and the TL fraction (which contains the ^{3}H internal standard) was eluted with 10 ml of ethyl acetate/benzene (1:20). The column was washed with an additional 10 ml of ethyl acetate/benzene (1:20) and the testosterone fraction was obtained with 10 ml of ethyl acetate/benzene (2:3). The TL fraction was transferred to a screw cap test tube (16 × 100 mm) equipped with a Teflon liner and evaporated under N_2, and the residue was dissolved in 100 μl of benzene, 900 μl of methanol, 100 μl of 10% aqueous potassium carbonate, and heated overnight at 50°C. Samples undergoing mock saponification were treated in the same manner but the potassium carbonate was omitted. Afterward, 100 μl of 9% aqueous acetic acid was added and any residual ester was removed by extraction with 2 ml of isooctane. Testosterone esters partition in the isooctane, and testosterone partitions in the aqueous methanol. The hydrocarbon layer was discarded and 900 μl of water was added to the aqueous methanol layer. The alcohol was removed under N_2 and the aqueous residue was extracted twice with 5 ml of ethyl ether. The ether extracts were combined and evaporated under N_2, and the residue was transferred to a test tube (12 × 75 mm) with several washings (total < 0.5 ml) of acetonitrile. After the organic solvent was evaporated, the residue was dissolved with vigorous Vortex mixing in 150 μl of human serum that had been stripped of endogenous steroid with dextran-coated charcoal (26). We have found that dissolving the residue in steroid-free serum and then analyzing for the steroid by using a nonextraction RIA decreases the blank. However, this step is not absolutely necessary. An aliquot of 30 μl of serum was assayed to determine the recovery of the ^{3}H internal standard, usually ≈40%. Samples in which the recovery was <25% were not used. The unesterified testosterone fraction, obtained from the alumina column with ethyl acetate/benzene (2:3), was evaporated under N_2 and then dissolved in steroid-free serum (recovery ≈ 70%). Both the TL (hydrolyzed) and testosterone fractions were analyzed by RIA: two aliquots of the serum (50 μl each) were analyzed directly by using a nonextraction ^{125}I RIA for testosterone CAC-TKTT (Diagnostics Products, Los Angeles). The commercial RIA is described by the manufacturer as specific for testosterone—that 5α-dihydrotestosterone cross-reacts only negligibly (which we confirmed). In the assay, 10 pg of testosterone displaces ≈20% of the bound tracer. The blank (no tissue) carried through the entire procedure was generally 7–10 pg. The results of the RIA were corrected for the blank and for recovery of the internal standard and normalized for the size of the aliquot and the weight of the tissue. They are reported as pg, molar equivalents, of testosterone (pg T equiv) per g of tissue.

To verify that the alumina chromatography eliminates testosterone from the nonpolar TL fraction, two experiments were performed. In one, 100,000 cpm of [^{3}H]testosterone was added to 250 mg of fat and the amount of radioactivity contaminating the TL fraction was measured: it was ≈100 cpm, or 0.1%. In another experiment, 10 ng of testosterone was added to 250 mg of female fat and the TL was analyzed. There was no measurable testosterone in the TL fraction (see Table 1). When the fat samples from female rats or long-term castrated males (>2 weeks) were analyzed by this protocol, the amount of TL measured was not significantly different than the blank (see Fig. 2) and in most experiments it was the same as the blank. When TL in fat samples from male rats was analyzed, significant amounts were found (see Table 1 and Fig. 2). If the TL fraction was not saponified (mock procedure) no testosterone was found (see Table 1). We assayed 10 ng of testosterone stearate directly by the RIA, and none was detected (data not shown). The testosterone ester does not cross-react in the assay unless it is hydrolyzed. The accuracy with which this assay measures TL was determined by adding various amounts (100–1500 pg T equiv) of exogenous testosterone stearate to ≈250 mg of female rat fat. The TL was measured as described and the results including the 95% confidence limits are shown in Fig. 1.

To confirm the RIA identification of testosterone in the TL fraction, fat samples from male and long-term (>2 weeks) castrated male rats were analyzed by GC/MS. One gram of each (divided into four separate 250-mg samples) was extracted and saponified as described above. After the addition of acetic acid and extraction with isooctane, the methanolic solutions were applied to C_{18} SPICE columns (Analtech). The columns were washed with 5 ml of methanol/water (1:1), after which the testosterone was eluted with 5 ml of methanol/water (7:3). The four samples were combined, the methanol was removed under N_2, and the aqueous residue was extracted twice with 2 vol of ether. The ether layers were evaporated under N_2 and the residues were dissolved in benzene.

The testosterone fractions were derivatized before analysis by conversion of the C-3 carbonyl group to a methyloxime and the 17β-hydroxyl group to a trimethylsilyl ether (27) as follows. The samples were dried under N_2, dissolved in 50 μl of a 2% solution of methoxyamine hydrochloride in pyridine (Pierce), and heated for 1 hr at 60°C. After removal of pyridine under N_2, 50 μl of trimethylsilylimidazole (Pierce) was added and derivatization was allowed to proceed for 1 hr at 100°C. Excess reagent was removed by diluting the sample with 1 ml of cyclohexane and passing it through a short column (in a Pasteur pipette) of Lipidex 5000 (Packard) equilibrated with cyclohexane. The testosterone derivative was eluted with 2.5 ml of cyclohexane. The solvent was evaporated under N_2, the resulting residue was dissolved in 20 μl of cyclohexane, and 2 μl was injected into the GC/MS.

The GC/MS analysis was carried out with a DB 1 column (15 m) (J & W Scientific, Rancho Cordova, CA) housed in a Hewlett–Packard 5790 gas chromatograph attached to a 5970 mass spectrometer (Hewlett–Packard). The sample was injected by splitless injection with the oven temperature remaining cool (50°C). After 3 min, the oven temperature was increased to 230°C at the rate of 27°C/min. The oven temperature was then raised by 2°C/min to a final temperature of 300°C. The eluent was analyzed by selected ion-monitoring using the parent ion (M^+) of testosterone methyloxine tri-

methylsilyl ether (m/z 389) and fragments m/z 358 (M-OCH_3) and m/z 125 (A-ring fragmentation).

RESULTS

The procedure designed to measure TL in various tissues quantifies the testosterone released by saponification from the nonpolar, TL, fraction. An internal standard of [^{3}H]testosterone stearate is added as a representative ester of TL to correct for procedural losses. The tissue is extracted and the extract is chromatographed on a column of alumina in order to obtain the nonpolar TL fraction free of testosterone (see above). The TL fraction is then saponified with potassium carbonate, partitioned between aqueous methanol and hexane to remove unhydrolyzed steroid, and analyzed with a RIA for testosterone. The results are corrected for the recovery of the ^{3}H internal standard. The validity of the procedure was tested in several ways. We showed that this procedure eliminates testosterone from the TL fraction. When [^{3}H]testosterone was added to fat, negligible radioactivity was found in the TL fraction. Furthermore, when 10 ng of testosterone was added to female fat (equivalent to 40 ng/g, an amount far in excess of endogenous testosterone) none of the added testosterone was measured in the TL fraction (Table 1). When fat from male rats was analyzed by this procedure, with the exception that a mock saponification was carried out in which potassium carbonate was omitted from the incubation, no assayable testosterone was found in the TL fraction (Table 1). This was expected since testosterone esters are not measured in the RIA unless first hydrolyzed to testosterone. When various amounts of testosterone stearate were added to fat and then carried through the entire procedure, including saponification, the exogenous ester was accurately detected (Fig. 1). A variety of tissues from male rats was analyzed for TL by this assay. As shown in Table 1, most tissues contain only negligible amounts of saponifiable testosterone in the TL fraction. This includes serum in which a larger sample volume, 1 ml, was analyzed in order to increase sensitivity. However, male fat contains significant amounts of TL: the TL in fat was assayed in intact males, in males that had been castrated for periods of >2 weeks, and in females. The results of this experiment are presented in Fig. 2. The amount of TL measured in these experiments (in T equiv per g of tissue ± SEM) is as follows: males, 2400 ± 174; females, 54 ± 14; long-term castrated males, 106 ± 45. In the experiments shown in Fig. 4 (the normal male is at time 0), the TL in male fat averaged 2700 ± 199. In all experiments on intact male rats, the TL in fat ranged from 1100 to 4100 pg T equiv per g. Thus, there is a relatively large amount of TL in the fat of the intact male but not in the fat of the female or the long-term castrate. While there may be some TL in both of the latter groups, the amount is below the sensitivity of the assay (see *Materials and Methods*). For comparison, in the experiments shown in Fig. 4, testosterone in male fat was also measured, averaging 9700 ± 1100 pg/g.

TL was also detected in the testes (Table 1), where it is 4000 pg T equiv/g. Since the testes have very high levels of testosterone, we again showed in this tissue that the TL was not caused by contamination with the endogenous testosterone. The TL fraction was put through a mock hydrolysis (without potassium carbonate) and then analyzed. There was no testosterone in the nonhydrolyzed TL fraction from testes (Table 1).

The hydrolyzed TL fraction isolated from male rat fat was also analyzed by GC/MS in order to confirm that the immu-

Table 1. TL in tissues of rat

Tissue	TL, pg T equiv/g
Male fat*	2400 ± 174
Male fat (nonsaponified)†	ND
Female fat	ND
Female fat + testosterone (40 ng/g)‡	ND
Testes§	4025 ± 537
Testes (nonsaponified)†	ND
Male serum, female serum, male brain, male liver, male muscle, male spleen	ND

ND, nondetectable values ranging from 0 to ≈150 pg/g. Each tissue was analyzed in at least three different assays. Data for TL are means ± SEM expressed as pg T equiv per g of tissue.
*From data in Fig. 2.
†The TL fraction was isolated as usual and then subjected to a mock saponification from which potassium carbonate was omitted.
‡Testosterone, 10 ng per sample, was added to ≈250 mg of fat.
§Mean of four experiments on a total of eight different samples: range, 1626–5728.

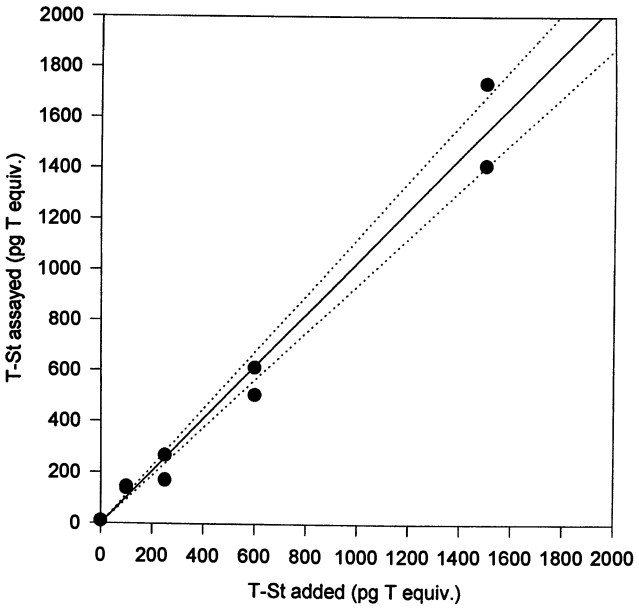

FIG. 1. Quantification of testosterone esters in fat. Various concentrations of testosterone 17-stearate (T-St) were added to 250-mg portions of female rat fat. The fat was extracted and the TL fraction was isolated and saponified; testosterone was measured by RIA as described in the text. Individual samples are shown. Dotted lines represent 95% confidence limits.

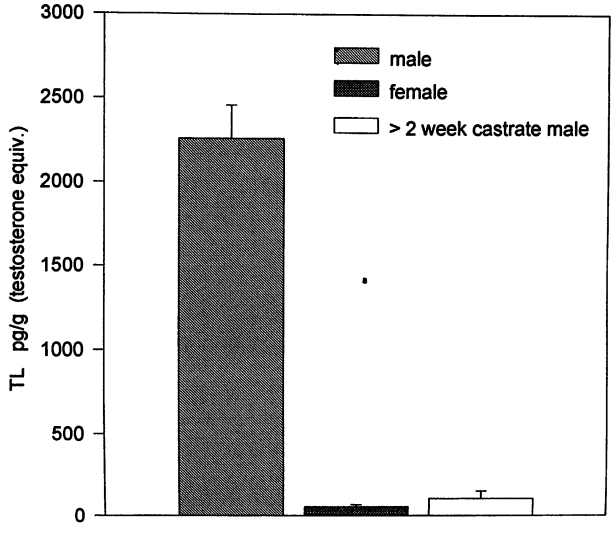

FIG. 2. TL in adipose tissue of rat. Samples of fat (≈250 mg) were analyzed for TL by RIA of the saponified TL fraction. Results are means from ≥10 separate experiments. Total number of fat samples analyzed are as follows: male, n = 29; female, n = 22; castrate, n = 16. Error bars are SEM.

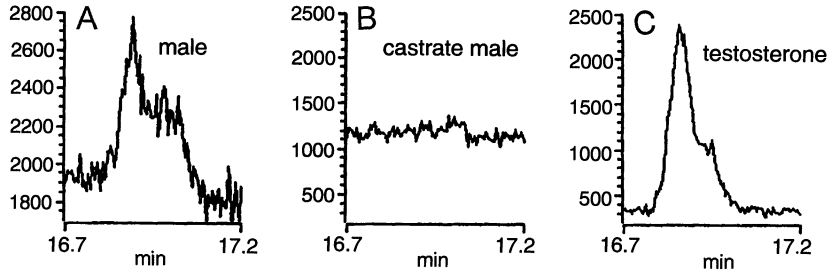

FIG. 3. GC/MS of saponified TL fraction from rat fat. Partial chromatograms of the $[M]^+$ (m/z 389) of testosterone methoxime trimethylsilyl ether. Samples of fat (1 g) from intact (*A*) and long-term castrated (*B*) male rats were extracted, saponified, and converted to the methyloxime trimethylsilyl ether as described in the text. The chromatogram (*C*) is testosterone run simultaneously as a reference. Analysis was performed on samples in *A* and *B* with aliquots that were representative of identical portions of tissue (as determined by recovery of the ^3H internal standard). In each panel the ordinate is the selected ion, m/z 389. The two incompletely resolved peaks at 16.9 and 17.0 min represent the *syn* and *anti* forms of the methyloxime derivative.

noassayable material is testosterone. The TL fraction from a normal male and a long-term male castrate was converted to testosterone by saponification, purified by chromatography on a C_{18} column, derivatized to form the methyloxime trimethylsilyl ether derivative, and then analyzed by single ion monitoring GC/MS: at m/z 389 parent $[M]^+$, at m/z 358 $[M-OCH_3]^+$, and at m/z 125 (A-ring fragmentation). The saponified TL fraction from the intact male rat gave a response for all three ions at the correct retention time for the methoxylamine trimethylsilyl ether derivative of testosterone. The partial ion chromatogram (m/z 389) of a standard of testosterone derivatized in the same manner and the two fat extracts are shown in Fig. 3. The two unresolved peaks at 16.9 and 17.0 min, observed with authentic testosterone and the male fat extract, represent the *syn* and *anti* forms of the methyloxime derivative (27). These peaks are not present in the saponified TL fraction of fat from the long-term castrated male rat.

Since steroidal esters are long-lived, presumably because they are protected from metabolism (9), we performed an experiment to compare the kinetics of the disappearance of TL and testosterone from fat after castration of male rats. As shown in Fig. 4*A*, almost all of the testosterone in the fat disappeared by 3 hr after castration and by 6 hr there was no longer any measurable testosterone in the fat. To the contrary, at those times (Fig. 4*A*) and even after an entire day (Fig. 4*B*), TL levels did not decline appreciably. Only after 48 hr of castration is a decrease in the concentration of TL noticeable. The TL levels in fat declined slowly thereafter, until 10 days when only very low levels were found.

DISCUSSION

These experiments show that TL exists in the fat and testes of the male rat. Although the structure of TL is not definitively proven, it is highly likely that TL is a heterogeneous family of fatty acid esters of testosterone: its physicochemical characteristics are the same as the fatty acid esters, and similar lipoidal derivatives of other steroids have been shown to be fatty acid esters (3, 6, 15–17, 28). The evidence that testosterone is released by hydrolysis of the nonpolar TL fraction from male rat fat rests on strong evidence, both immunological and spectral. The immunological (RIA) data show little if any TL in the long-term castrated male or in the female rat (Fig. 2). This is consistent with a metabolite of testosterone. Most compelling is the GC/MS analysis, which shows testosterone in the hydrolyzed TL fraction of male fat but not the long-term castrated male rat.

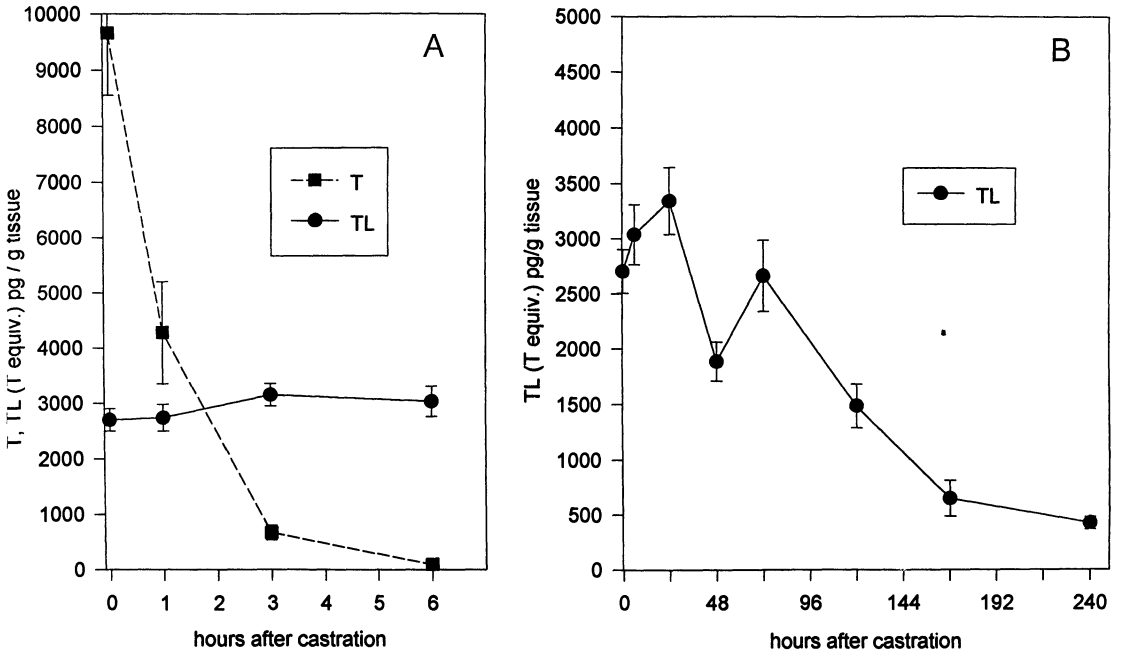

FIG. 4. Effect of duration of castration on concentration of testosterone (T) and TL in male rat fat. Each point is the mean of at least three separate determinations. Total number of different fat samples analyzed for each point is ≥5. Error bars are SEM.

There is no TL in the other tissues tested (Table 1), including muscle and blood. There is also no TL in the brain. This is of interest because the first demonstration of the fatty acid esterification of a steroid hormone was the esterification of testosterone by a brain preparation (29). In this study, while we did not find TL, we did find relatively large amounts of testosterone in the brain (5900 ± 1000 pg/g; mean ± SEM). Thus, although both the substrate and the enzymatic capacity to esterify testosterone is in the brain, there is no TL. Although this might appear to be contradictory, there are several reasons why this might occur, including rapid hydrolysis of TL; the acyltransferase enzyme found in the *in vitro* experiments with brain has a high K_m (low affinity) for testosterone, insufficient for the *in vivo* concentration; or the enzyme and substrate are present in different regions of the brain. Regardless of the reason, the synthesis or accumulation of TL is obviously more complex than might be otherwise apparent. The tissue distribution of TL is consistent with our studies of the E_2 esters in humans in which we found that there is very little in muscle or blood and that most was present in fat (18). It does not agree with the findings of a substance with the properties of TL in the blood of men (19, 20). The finding of high levels of a compound with the properties of testosterone esters in blood is unusual since the hydrophobic esters of steroids do not appear to be secreted into blood (18, 30). Although fairly high concentrations of some steroid esters circulate in blood, they are the esters of the Δ^5-3β-hydroxysteroids pregnenolone and dehydroisoandrosterone (14). They are formed in blood by lecithin:cholesterol acyltransferase (LCAT) for which they are good substrates (14, 31). E_2 esters are in also blood, but E_2 is a poor substrate for the enzyme, and this is probably the major reason why only very low levels are present. However, testosterone is not esterified at all by LCAT (14). As pointed out above, the compound measured in human male blood (19, 20) did not disappear after long-term castration (surgical or pharmacological) or even complete inhibition of steroidogenesis with aminoglutethimide, and its level in blood was inversely proportional to that of testosterone (20). There are obvious differences between those studies and this one, especially the species used and many of the techniques, and so it is not possible to conclude that TL is not in human blood. Obviously, the confirmation of the presence of TL in human blood requires direct experimentation. However, whatever that substance is, its biological properties are very different than those that we found for TL in rat fat (see Fig. 4).

These studies demonstrate the presence of a nonpolar metabolite of testosterone, TL, in the fat and testes of the male rat. This metabolite is extremely long-lived when compared to the parent steroid, testosterone (Fig. 4). Prolonged biological half-life ($t_{1/2}$) is linked to increased androgenic potency, as evidenced by the synthetic androgenic esters that are used therapeutically (23). This relationship of biological $t_{1/2}$ and potency has been confirmed with the naturally occurring E_2 17-fatty acid esters, LE_2, which because they are protected from metabolism have a considerably extended biological life (9) and are therefore remarkably potent estrogens (10, 11). Likewise, it would be expected that because the endogenous testosterone esters, TL, are long-lived, they are highly potent androgens. It is very interesting that in the relatively long period after castration, when the levels of testosterone are undetectable, sizable quantities of TL are still present in the fat (Fig. 4). At these times, although testosterone is not being synthesized by *de novo* steroidogenesis, the androgen can still be produced, not by the steroidogenic enzyme that synthesizes C_{19} steroids, the 17-hydroxylase, but by enzymatic hydrolysis of TL. While the amount of testosterone that is produced may be small if diluted into the entire body pool, locally sizable amounts may be secreted. We hypothesize that stimulation of specific androgen target tissues can occur by a paracrine mechanism through which TL in neighboring fat supplies testosterone through the action of esterase(s). Hormonal communication from the target tissue to the fat could signal adipose cells to synthesize or activate the esterase, leading to the release of androgen at times when testicular production or blood levels of testosterone are low. Since, in this view, the androgenic stimulation would be only through local fat and not the circulation, specificity of the target tissues is possible. Regardless of how TL may act, the existence of this highly unusual, long-lived metabolite of testosterone undoubtedly has important ramifications for our understanding of the physiology of the sex steroids.

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