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Metabolism of testosterone and its ester derivatives in organotypic coculture of human dermal fibroblasts with differentiated epidermis

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Abstract

The metabolism of testosterone (TS) and its 17-O-acyl derivatives (acyl = acetyl, benzoyl and hemi-succinoyl) was studied using radioactive compounds in organotypic coculture of human dermal fibroblasts (living skin equivalent, LSE). When TS was applied to the epidermal-side of LSE in a small volume of acetone solution, both 5α-reduced and 17-dehydrogenated metabolites were observed in the dermal-side culture solution, though the formation of the 5α-reduced metabolites, dihydrotestosterone (DHT) and dihydroandrosterone (DHA), depended greatly on the culture conditions. The metabolic activity of LSE for testosterone was higher than that of excised hairless-rat skin. The metabolism of ester prodrugs of TS in LSE was dependent on their physicochemical properties and susceptibility to enzymatic hydrolysis. Application of acetyl-TS and benzoyl-TS resulted in a high formation of the 17-dehydrogenated metabolite, androstenedione (ADO), though a very small amount of the prodrug was observed in the dermal side. Succinoyl-TS, a hydrophilic ester with very low susceptibility to hydrolysis, was quite resistant to both 5α-reduction and 17-dehydrogenation, and more than 90% of the radioactivity appearing on the dermal side was from the prodrug itself and from TS. The hydrophilic and enzymatically stable TS derivative may be a good candidate compound with which to administer TS transdermally.

Keywords: Testosterone; Metabolism; 5α-Reductase; Prodrug; Living skin equivalent (LSE); Transdermal delivery

1. Introduction

Transdermal delivery of testosterone to hypogonadal men (Korenman et al., 1987; Mazer et al., 1992) is currently recognized as an alternative method to conventional intramuscular injection of ester suspensions or oral administration of alkylated derivatives. Though the transdermal route would provide controlled delivery of many drugs, complex biotransformations of testosterone including 5α-reduction and 17-hydrogenation have promoted interest in a transdermal first-pass effect

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for this drug. Abnormally large formation of dehydrotestosterone, a product of 5α-reductase, was clinically observed when a trans-scrotal delivery patch was applied to hypogonadal men (Korenman et al., 1987), and this has raised some concern regarding the potential over-stimulation of dihydrotestosterone sensitive tissues such as the prostate.

Metabolic studies employing excised human skin (Rongone, 1966; Bingham and Shaw, 1973) and isolated skin tissues (Ebling and Randall, 1985) have shown that 5α-reduced metabolites including dehydrotestosterone are the major metabolites, though complexity of testosterone metabolism in the various contributing tissues of the skin was also observed. Investigation of various metabolites and evaluation of derivatives for the transdermal route will inevitably require simplified in vitro studies. Studies using monolayers of human dermal fibroblasts (Schweikert et al., 1982; Milewich et al., 1986) and keratinocytes (Milewich et al., 1988) have demonstrated a degree of metabolic similarity of skin and provide homogeneous viable cell populations in a controlled environment. An organotypic coculture of human dermal fibroblasts in a collagen-containing matrix, living skin equivalent (LSE), has recently been recognized as a simple model to simulate the metabolism of living human skin (Bell et al., 1991). The LSE has two viable and metabolically active cell populations (fibroblasts and keratinocytes) which have been shown to possess biosynthetic properties that more closely approximate their in vivo counterparts, human skin, than do those in monolayer culture (Nusgens et al., 1984).

In this report, we carried out a comparative study on testosterone metabolism by using LSE and excised hairless-rat skin. After confirming the usefulness of LSE for the metabolic study, the permeation and metabolic characteristics of several testosterone ester prodrugs were evaluated in the LSE system.

2. Experimental

2.1. Analytical

A high-performance liquid chromatography system (LC-10AS, Shimadzu) equipped with a variable wavelength detector (SPD-10A, Shimadzu), a 20-μl fixed loop injector (Model 7125, Rheodyne, USA), and a chromatograph terminal (CR-5A, Shimadzu) was used. Chromatography was performed on a Lichrosorb RP-18 column (4 × 300 mm, Merck, Germany) at 40°C, elution was carried out at 1.0 ml/min and absorbance was monitored at 245 nm. Radioactivity measurements were done by a liquid scintillation counter (LSC-700, Aloka).

2.2. Materials

Reference steroids were obtained from Sigma Chemical Co. (CEA AB, Sweden). 5α-Dihydrotestosterone (DHT) was purified by recrystallization from ethanol, and other references were used as obtained. Radioactive steroid [4-^{14}C]testosterone (SA 57.3 mCi/mmol) was obtained from Du Pont (Boston, MA, USA). Non-radioactive derivatives of testosterone were synthesized in a pyridine solution with a corresponding acid anhydride, and purified by silica gel column chromatography. Radioactive esters were synthesized using [4-^{14}C]testosterone (SA 57.3 mCi/mmol) at 1 μmol scale in a 0.2 ml anhydrous pyridine solution. After acylation with a corresponding acid anhydride, the reaction mixture was chromatographed on thin-layer chromatography (50 × 200 mm, HPTLC Kieselgel, E. Merck, Germany). The corresponding spot was detected by UV and collected in a glass tube by scraping. Extraction with ethanol gave a solution of radioactive derivatives. The purity of the derivatives was determined by HPTLC and was at least 96%, based upon the radioactivity recoverable at the R_f value for the derivative on the HPTLC plate.

2.3. Tissues and incubation conditions

The fibroblast and keratinocyte strains used in the fabrication of LSE were derived originally from human foreskin and propagated in monolayer culture. LSEs were manufactured at the

Tsuruga Institute of Bio-technology, Toyobo Co., Shiga, Japan, and part of those used in this experiment were generously provided by the Toyobo Co. The morphological and biochemical properties of LSE were reported previously (Bell et al., 1991).

Male hairless-rats (200–220 g) were purchased from Tokyo Laboratory Animals Co., and were sacrificed to obtain the entire abdominal skin.

The skin or LSE was homogenized with pH 7.4 isotonic buffer (0.1 M) containing sucrose at 0°C to give a concentration of 1.0% (w/v). The homogenates were centrifuged at 1000 × *g* for 20 min, and the resulting supernatants were stored at −80°C until use. Susceptibility of ester derivatives for enzymatic hydrolysis in the homogenates was determined by the method reported previously (Kawaguchi et al., 1985). The enzymatic activity for the hydrolysis was not changed before and after the freezing as reported previously (Kawaguchi et al., 1990).

2.4. Metabolic studies

The metabolic study was carried out as reported (Ernesti et al., 1992) with minor modifications. Briefly, the radiolabeled testosterone or its derivative was dried and reconstituted in acetone containing 5.2 μmol of unlabeled compound and 52 nmol (3 μCi) of labeled corresponding compound per 80 μl of acetone. Prior to application of a test compound, the dermal portion of the LSE was rested on a 3-μm pore size polycarbonate membrane (Transwell, Costar Co., MA, USA) in contact with 1.5 ml of assay medium (DMEM/Ham's F-12, 1:1 cell culture media supplemented with 50 μg/ml gentamicin sulfate), while the epidermis was cultivated at an air-liquid interface. After 1 h incubation at 37°C in a humidified incubator gassed with 5% CO_2, a polyethylene ring (0.8 cm^2 internal area) was affixed to the epidermal surface with silicone sealant, and the medium was replaced by 1.5 ml of a modified medium (1:1 mixture of DMEM/Ham's F-1 cell culture medium with reduced bicarbonate (0.35 g/l) supplemented with 1 μg/ml gentamicin sulfate and 10 mM HEPES buffer); this enabled assays to be performed in the absence of gassed CO_2. At the start of the study, 80 μl of the acetone solution were added to the interior of the polyethylene ring, and the solvent was evaporated under a normal atmosphere for 5 min. After the surface was dried, this assay system was allowed to incubate at 37°C in a humidified normal atmosphere. Fresh medium replaced the assay medium (receiver or dermal side solution) at 1, 2, 4, 8, and 24 h, and each sample medium was extracted twice in chloroform (1.5 ml). The chloroform samples were combined and dried under nitrogen gas flow, and the residue was reconstituted with a small volume of chloroform and applied on TLC (HPTLC, 200 × 200 mm, Alufolien Kiesegel 60 F_{245}, E. Merck, Germany). The TLC plates were developed with a mixture of dichloromethane and ethylether (9:1). After the plate had been completely run, it was sprayed with an enhancing fluor (NEF-970, NEF Research Products, Boston, MA) and dried for 10 min. Fluorography was done at −80°C for 10 days using an X-ray film for fluorography (Hyperfilm, Amersham International plc., CEA AB, Sweden).

For quantification of each compound, corresponding spots were scraped by referencing their autoradiography, and counted in a liquid scintillation counter. Formation of each metabolite was compared as its percent of the total radioactivity permeated.

The study using hairless-rat abdominal skin was done with a two-chamber diffusion cell which had an available diffusion area of 0.95 cm^2 and a half-cell volume of 2.3 ml. Cell sets were kept at 37°C in a water bath, and both sides of the skin were exposed to 40% poly(ethylene glycol) solution. The donor (epidermal side) solution contained 0.1 μmol (6 μCi) of [4-^{14}C]testosterone and unlabeled testosterone (10 μmol). A sample of whole receptor solution was removed at 1, 2, 4, 8, and 24 h, and refilled with fresh solution. The collected receptor solution was extracted twice in chloroform (2 ml), and analyzed as described above.

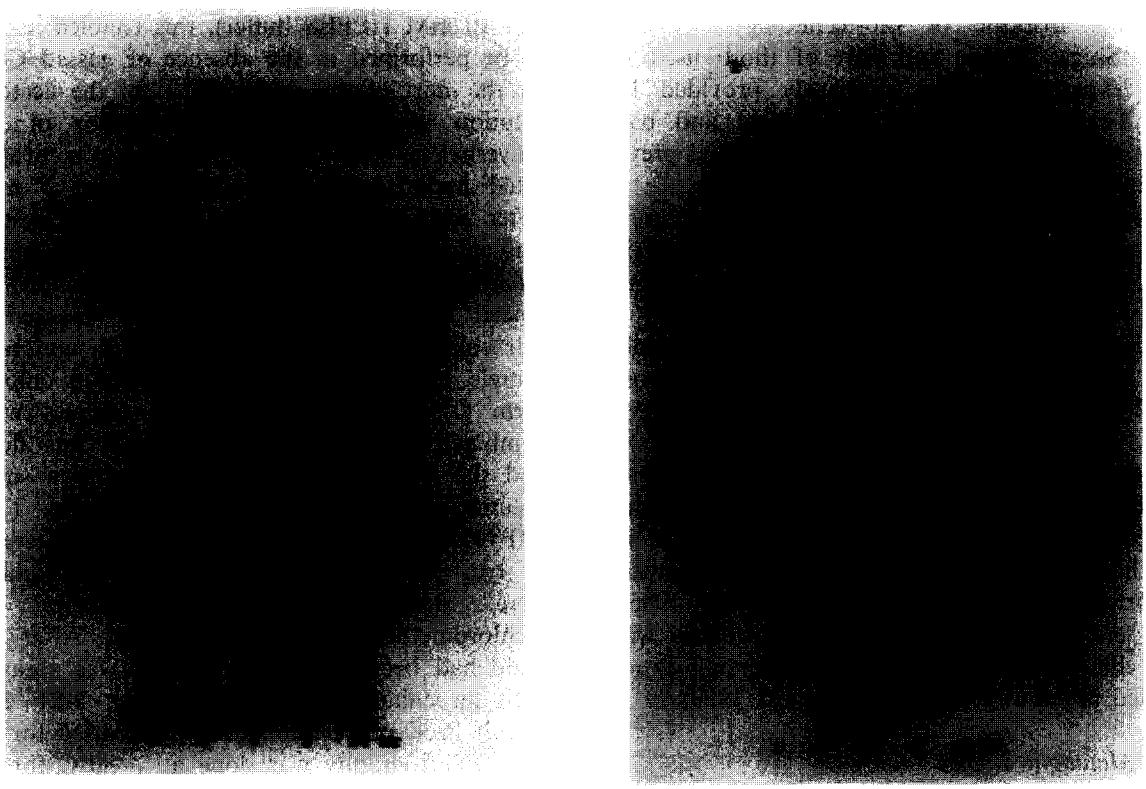

Fig. 1. Autoradiographic demonstration of the radioactive metabolites on TLC of [4-^{14}C]testosterone permeated through LSE or hairless-rat skin. (A) LSE; (B) hairless rat skin; (C) LSE with water as the receiver solution instead of the culture medium. 1, androstandione; 2, androstenedione (ADO); 3, dihydrotestosterone (DHT); 4, androsterone; 5, testosteron (TS); 6, dihydroandrosterone (DHA)

3. Results and discussion

3.1. Metabolism of testosterone

Testosterone metabolism in LSE was determined by thin-layer chromatographically separated radioactivity of permeants through the LSE layer. Six areas of radioactivity could be identified as testosterone and its metabolites. A typical autoradiograph of the chromatogram is shown in Fig. 1A. The formation of metabolites including 5α-reduced metabolites was more evident in the LSE than in hairless rat skin (Fig. 1B). The formation of 5α-reduced metabolites was greatly inhibited when the receiver solution was changed from the culture medium to water during the penetration experiment (Fig. 1C), though the formation of androstenedione, a product of 17β-hydroxysteroid dehydrogenase, was little affected. Ernesti et al. (1992) studied testosterone metabolism using a similar human skin culture and reported high 5α-reductase activity. Our results also suggest that LSE maintains higher activity of steroid metabolic enzymes than excited animal skin, though 5α-reductase activity is quite sensitive to the experimental conditions used. Quantification of three metabolites, androstenedione, dihydrotestosterone (DHT), and dihydroandrosterone (DHA, a metabolite of DHT), is presented in Fig. 2 as the percent of total radioactivity penetrated at 2 and 8 h. Formation of their 5α-reduced metabolites, DHT and DHA, ac-

Fig. 1C.

counted for 5–6% of the permeants, this value being consistent with the result obtained in a clinical study using the trans-scrotal testosterone system reported by Korenman et al. (1987).

3.2. Metabolism of testosterone prodrugs

Since the above results showed that the LSE simulates certain aspects of human skin metabolism for testosterone, the metabolism of several testosterone prodrugs was studied using the same experimental conditions. The prodrugs, 17-O-esters of testosterone, showed different physicochemical properties and susceptibility to enzymatic hydrolysis (Table 1). Such properties as partition coefficient, molecular weight and susceptibility to esterase may affect their permeation through the LSE and/or the metabolism. Fig. 3 shows the permeation-time profiles of the prodrugs and their metabolites through the LSE membrane. The profiles were almost linear for the initial 4 h except for that of TS-succinate, and no lag-time was observed for any compound. The permeation rates largely decreased after 8 h compared with those during the initial 4 h. The prodrugs were applied as a small volume of acetone

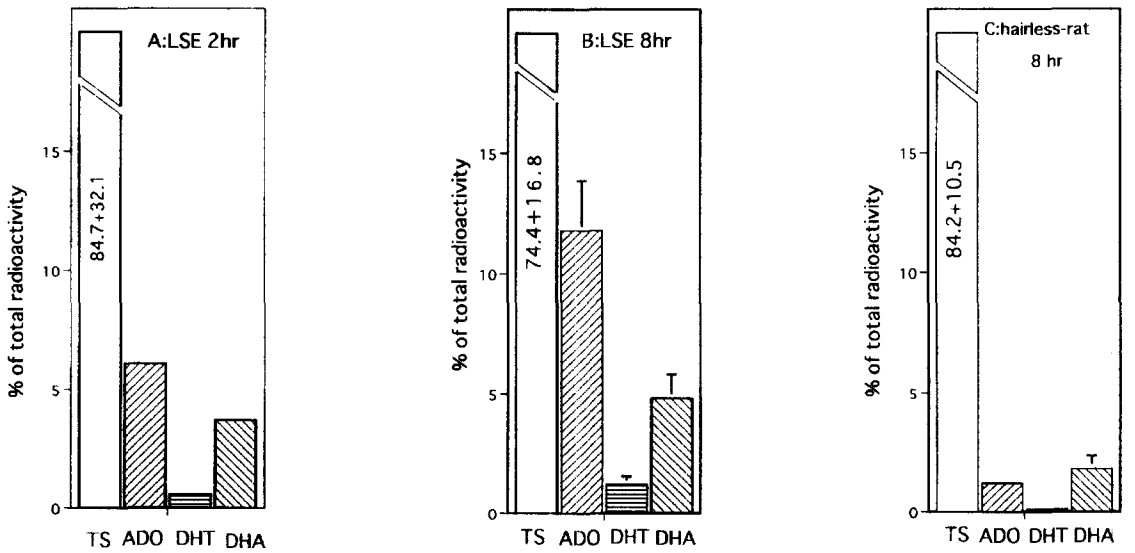

Fig. 2. Relative radioactivity of androstenedione (ADO), dihydrotestosterone (DHT), and dihydroandrosterone (DHA) to total radioactivity permeated through LSE at 2 h (A), LSE at 8 h (B), and LSE with water as the receiver solution instead of the culture medium at 8 h (C). The vertical bars represent the mean ± S.E.M. (n = 3).

Table 1
Physicochemical properties and susceptibility to enzymatic hydrolysis of 17-*O*-acyl-testosterone

Compound	Mol. wt.	m.p. (°C)	log PC[a]	Susceptibility[b]	
				Rat skin[c]	LSE[d]
Testosterone	288	155	3.7	–	–
Acetate	330	135	4.4	288	163
Benzoate	393	196	5.4	425	104
Succinate	388	192	3.3	47 000	19 000

[a]Partition coefficient between chloroform/water at 20°C.
[b]Half-life in solution at 37°C, min.
[c]1% Hairless-rat skin homogenate.
[d]1% LSE homogenate.

solution in this experiment, so their activity on the donor side does not seem to be constant, and this may have affected the permeation rates following the initial linear profiles. Total radioactivity permeated from TS-succinate and TS-acetate was one-half and one-third of that from TS (about 6 × 10^5 dpm, 0.47 mmol, at 8 h in Fig. 1A), respectively. Since susceptibility for the enzymatic hydrolysis is comparable between the prodrugs (Table 1), the higher total radioactivity permeated from TS-acetate than from TS-benzoate may indicate an appropriate partition coefficient of TS-acetate for the permeation through the LSE. Fig. 4 indicates the percent of the prodrugs and their metabolites of the total radioactivity on the receptor side after linear permeation (2 h after the application of prodrugs). Testosterone and androstenedione were the major permeants when

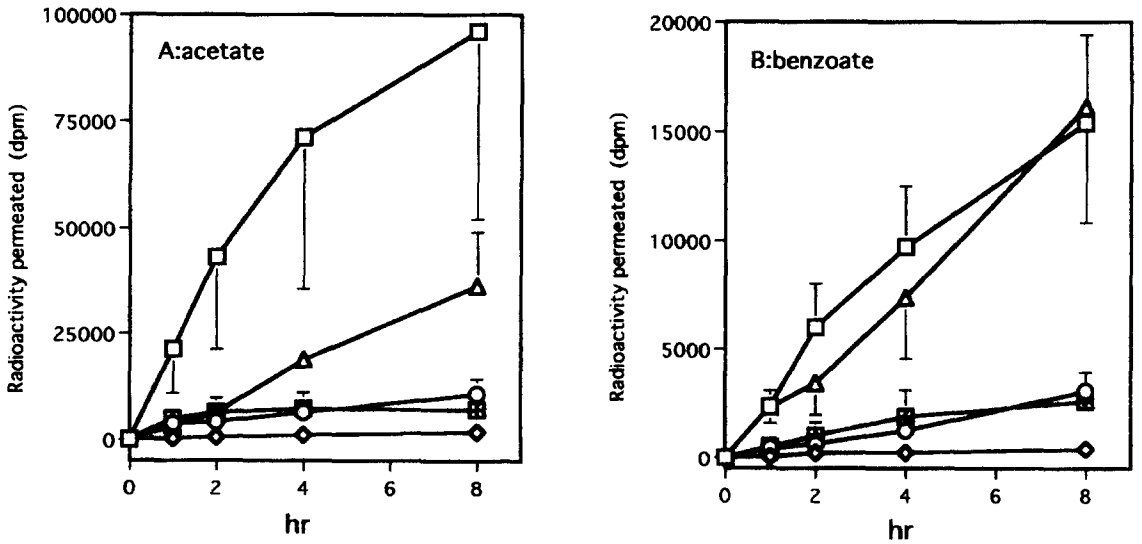

Fig. 3. Permeation profiles of prodrugs and their metabolites. (A) Acetate; (B) benzoate; (C) succinate. prodrug (⊕), testosterone (□), androstenedione (△), DHT (◇), dihydroandrosterone (○).

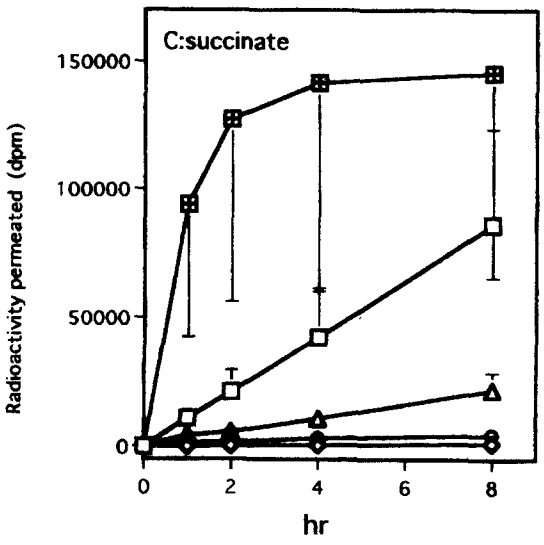

Fig. 3C.

TS-acetate or TS-benzoate was applied. Since both prodrugs were sensitive for the enzymatic hydrolysis to testosterone, only a small amount of the total radioactivity (9% and 8% of TS-acetate and TS-benzoate, respectively) was permeated as an intact prodrug. Amounts of the 5α-reduced metabolites at 2 h (DHT plus DHA), were 7.5 and 8% of the total radioactivity permeated when TS-benzoate and TS-acetate were applied, respectively. These values were higher than that of unmodified TS at 2 h (4.5%). Retention and/or distribution of the lipophilic prodrugs in the tissue (LSE) may increase the metabolite formation. Formation of 17-dehydrogenated metabolite, androstenedione, also increased to 13% and 25% of the total permeants of TS-acetate and TS-benzoate, respectively, while that of TS was 6%. This results was unexpected, since the 17-O-modifica-

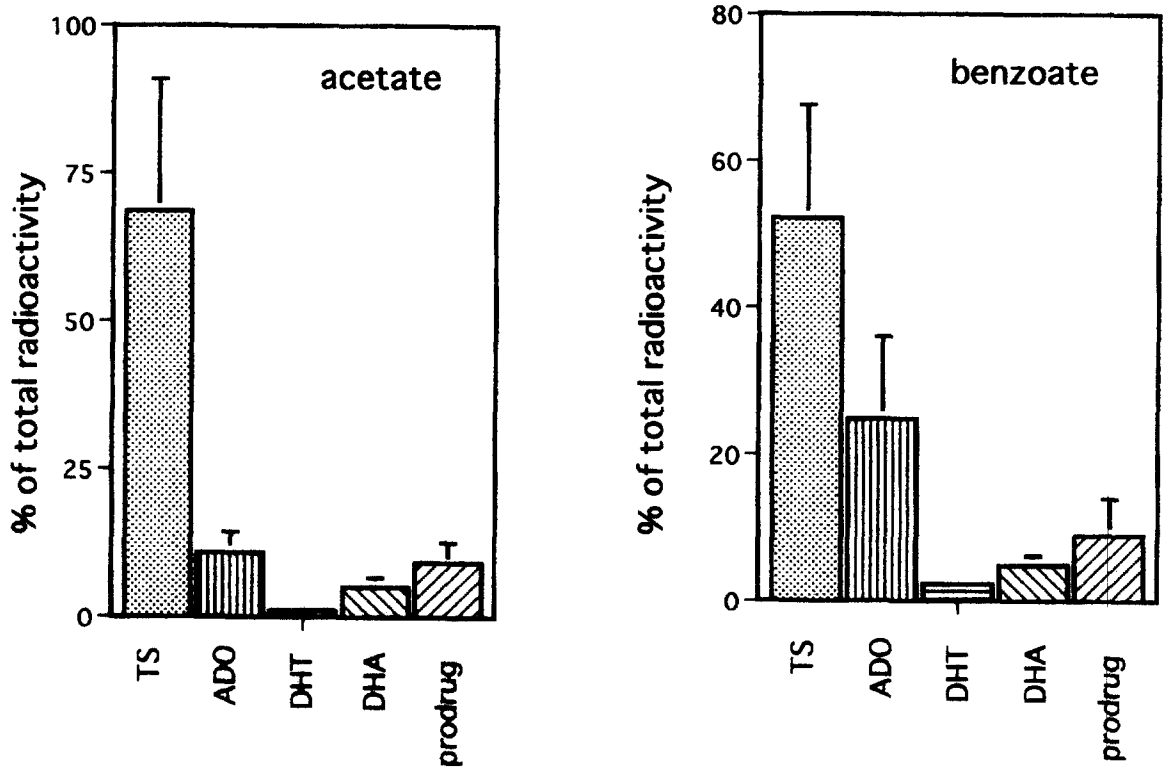

Fig. 4. Relative radioactivity of prodrugs, testosterone (TS), androstenedione (ADO), dihydrotestosterone (DHT), and dihydroandrosterone (DHA) to the total radioactivity permeated through LES. The vertical bars represent the mean ± S.E.M. (n = 3).

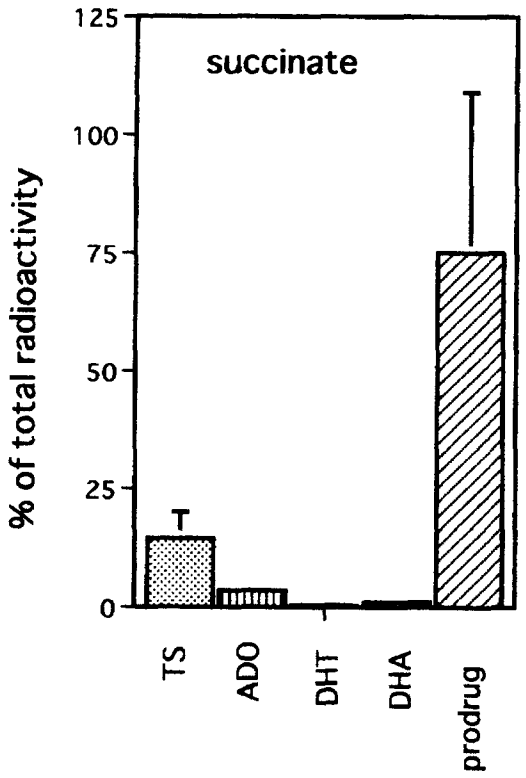

Fig. 4C.

tion was expected to inhibit 17-metabolism, and cannot yet be explained, though introduction of a certain lipophilic moiety on the 17-alcohol may affect the affinity of the compound to the enzyme(s) responsible for the dehydrogenation.

TS-succinate showed quite different characteristics than the above two prodrugs, including very low susceptibility to enzymatic hydrolysis and high hydrophilicity (Table 1). Metabolism of TS-succinate reflected its susceptibility against the enzymatic hydrolysis; more than 75% of the radioactivity permeated stemmed from the intact prodrug, and about 90% of the permeant was the prodrug and the parent drug. Contrary to TS-acetate and TS-benzoate, the formation of androstenedione was remarkably inhibited by the introduction of hemi-succinate at the 17-position, and only 4.5% of the total permeant was the 17-dehydrogenated metabolite. Formation of the 5α-reduced metabolites was also reduced to less than half (2.5% of the total permeants) compared with TS or other prodrugs. 17-O-modification with anionic hemi-ester may be effective in inhibiting 5α-reductase, though direct evidence of that was not obtained from this experiment.

We focused here on the metabolism of testosterone and its 17-O-esters in cultured human dermal fibroblasts, which can simulate human skin metabolism. Though further detailed experiments including long-term permeation or absorption studies must be made for a conclusive assessment, the better metabolic characteristics of 17-*O*-succinoyl-testosterone were shown in this experiment. The usefulness of LSE in simulation of human skin metabolism should also be studied for other drugs and under various experimental conditions.

Acknowledgements

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