Metabolism of Testosterone During *in Vitro* Transport Across CACO-2 Cell Monolayers: Evidence for β-Hydroxysteroid Dehydrogenase Activity in Differentiated CACO-2 Cells

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Testosterone has previously been used as a model compound for the determination of unstirred water layer thickness in the CACO-2 transport model. We have found, however, that testosterone is metabolized during *in vitro* transport across the CACO-2 cell monolayers. This suggests that testosterone is not an ideal model substance. Testosterone is metabolized to androstenedione, indicating the formation of 17-β-hydroxysteroid dehydrogenase by differentiated CACO-2 cells. No reverse metabolism is observed, thus androstenedione is considered superior to testosterone for determination of unstirred water layer thickness in the CACO-2 system. Permeability coefficients for testosterone and androstenedione obtained under identical transport conditions were 66 (±7) * 10^{-6} (n = 26) and 84 (±7) * 10^{-6} (n = 9) cm/sec, respectively. The unstirred water layer thicknesses at different agitation rates are determined for the CACO-2 transport model used in our laboratory utilizing androstenedione as a model compound. The system is capable of controlling the water layer thickness from about 200 to 1000 μm.

KEY WORDS: CACO-2; cell culture; unstirred water layer; metabolism; testosterone.

INTRODUCTION

The human intestinal cell line, CACO-2, cultured on microporous filter membranes, is extensively used as a model to study drug transport and metabolism in intestinal epithelium (1–4). It is well recognized that transport of lipophilic solutes across the cell monolayers is determined by at least two major diffusion barriers. One is the unstirred water layer at the interface between the bulk solution and the cell monolayer, and the other the cell membrane. When the unstirred water layer exerts a major portion of the total diffusional resistance, parameters such as permeability coefficients may be underestimated. Hence, for the characterization of a transport system, it is important to determine the thickness of the unstirred water layer. To this end, lipophilic compounds such as testosterone have previously been utilized (5–7).

We have found, however, that testosterone is being metabolized during transport across CACO-2 cell monolayers and hence may not possess ideal characteristics as a model substance for determination of unstirred water layer thickness in the CACO-2 model.

The purpose of this study is to identify and quantitate metabolite formation exhibited by CACO-2 cells on the genuine sex hormone testosterone. A further objective is to evaluate the impact of metabolism on the determination of unstirred water layer thickness based on transport of testosterone.

MATERIALS AND METHODS

Chemicals

Testosterone was purchased from Fluka, Switzerland. 5-α-Androstane-3,17-dione, 4-androstene-3,17-dione, androsterone, etiocholan-3-ol-17-one, estradiol, and estrone were purchased from Sigma, St. Louis, MO. All compounds were of analytical grade and no further purification was performed.

Cell Cultures

The CACO-2 cells (8,9) were obtained from the American Tissue Culture Collection (Rockville, MD) and maintained in tissue culture flasks (Greiner, Kremsmünster, Austria). Dulbecco's modified Eagle's medium containing 9% heat-inactivated fetal calf serum, 1.0% nonessential amino acids, benzylpenicillin (100 U/ml), and streptomycin (10 U/ml) was used as growth medium (Gibco, Middlesex, UK) and the cells were kept in an atmosphere of 5% carbon dioxide and 90% relative humidity.

The cells were grown on uncoated polycarbonate filter inserts (pore size, 0.4 μm; diameter, 24.5 mm) in six-well cluster dishes (Transwell, Costar, MA). Approximately 2 * 10^6 cells were added to each insert. The cells were fed every second day and the cell monolayers were used 21–30 days after seeding. Passage numbers 40–50 were used. The cells were mycoplasma negative (tests performed by Mycoplasma Laboratory, Statens Seruminstitut, Copenhagen, Denmark).

Transport Studies

Transport studies were performed in the filter inserts, which were kept at constant temperature (37°C) in an atmosphere of 95% oxygen and 5% carbon dioxide. Agitation of the cell monolayers was performed using a plate shaker with controllable speed (100–1000 rpm) and angle (1–5°) relative to horizontal position. Fifty to one hundred microliters of stock solutions of testosterone or estradiol in ethanol was added to 50.0 ml of transport medium (Hanks balanced salt solution), and after mixing, 2.50 ml was used in the apical compartment (the final concentration being 10^{-5} or 10^{-4} M). Three milliliters of transport medium was then added to the basolateral compartment. At predetermined time points samples of 50–100 μl were taken from the basolateral and the apical compartment.

The integrity of the cell monolayers was checked by measurement of the transepithelial electrical resistance (Mil-

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licell, Millipore, Bedford, MA) and by the use of the hydrophilic marker ^{14}C-polyethylene glycol (PEG) with a specific activity of 10 μCi/mg and a molecular weight of 4000 daltons (Amersham, UK). Five milliliters of scintillant (Ultima Gold, Packard, CT) was added to each test tube and the samples were counted in a liquid scintillation counter (Packard Model 2000).

Analytical Procedure

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Merck Hitachi pump (Model L-6200), a Merck Hitachi variable UV detector (Type L-4200), and a Merck Hitachi autosampler (Model AS-4000). Data acquisition and processing were performed using the Merck Hitachi HPLC-manager, Model D-6000. The analytical column was a reversed-phase ChromSep column (4.6 * 100 mm) packed with Microspher C18 (3-μm particles, ChromPack, The Netherlands) and protected with a precolumn (2.1 * 10 mm) packed with pellicular material (30- to 40-μm particles, ChromPack). The mobile phase consisted of 40% acetonitrile in distilled water and the flow rate was maintained at 0.90 ml/min. The effluent was monitored at 190 or 200 nm. The following retention times (min) and capacity factors were obtained: testosterone, 6.4 and 5.1; 4-androstene-3-17-dione, 8.5 and 7.1; etiocholan-3-ol-17-one, 14.0 and 12.3; 5-α-androstane-3,17-dione, 15.4 and 13.6; and androsterone, 16.5 and 14.7. Mass spectrometry was performed on a Finnigan MAT Model 4515B using an electron impact of 70 eV.

Data Treatment

The apparent permeability coefficient (P_{app}) were calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt * A * C_0 * 60} \quad (1)$$

where dQ/dt is the flux across the monolayer (nmol/min), A is the surface area of the membrane (4.7 cm^2), and C_0 is the initial drug concentration. In all runs the permeability coefficients were obtained on the basis of transport studies performed under "sink conditions," i.e., less than 10% transported.

RESULTS AND DISCUSSION

The integrity of the cell monolayers was checked by measuring the electrical transepithelial resistance before and after the transport experiments (Table I) and by using the hydrophilic marker ^{14}C-polyethylene glycol (^{14}C-PEG; Fig. 1). From Table I it is seen that no change in the transepithelial resistance is induced during the transport experiments (at agitation rates less than about 700 rpm), indicating the monolayer to be confluent and intact during the time course of the experiments. The resistance readings are within the range of values reported previously (1–3,10,11).

Table I. Transepithelial Resistance (R)[a] Before and After Transport Experiments

	$R(\Omega * cm^2) \pm SD\ (n)$	
	400 rpm	700 rpm
Before experiment	459 ± 56 (20)	432 ± 23 (10)
After experiment	451 ± 48 (20)	449 ± 70 (10)

[a] After subtraction of the intrinsic resistance of cell-free polycarbonate filters.

The time course of the percentage of ^{14}C-PEG transported from the apical to the basolateral compartment is shown in Fig. 1. It appears that the cell monolayers are impermeable to the hydrophilic macromolecule (MW 4000 D). Thus, only a small fraction (≈0.1%) is transported per hour. This value is somewhat higher than that reported by Artursson (2) but lower than that observed by Cogburn and co-workers (12). We consider the cell monolayer to be confluent and intact because of the observation that, without the cells present, more than 20% of ^{14}C-PEG is transported across the cell monolayer within 1 hr. Thus only 0.5% of ^{14}C-PEG is transported across the CACO-2 cells as compared to transport across the polycarbonate filters. Further, as pointed out by Artursson (2) the commercially available ^{14}C-PEG, to some extent, contains lower molecular weight fractions. The amount of these fractions may vary somewhat from one batch to another, explaining the slightly different results obtained in this study and the studies by Artursson (2) and Cogburn and co-workers (12).

In an effort to characterize the CACO-2 cell transport model used in our laboratory, we have performed penetration experiments using the genuine sex hormone testosterone as a model substance. Testosterone has previously been used with the aim of determining the unstirred water layer thickness in the CACO-2 model (5–7). The analytical procedure used in our studies is HPLC with ultraviolet spectroscopic detection (190 or 200 nm). The procedure allows determination of testosterone in the presence of various metabolites of the hormone. Chromatograms obtained from the basolateral compartment surprisingly contained an additional peak to testosterone. This peak was further found to increase in height with time during the transport experiments, suggesting metabolite formation during the time

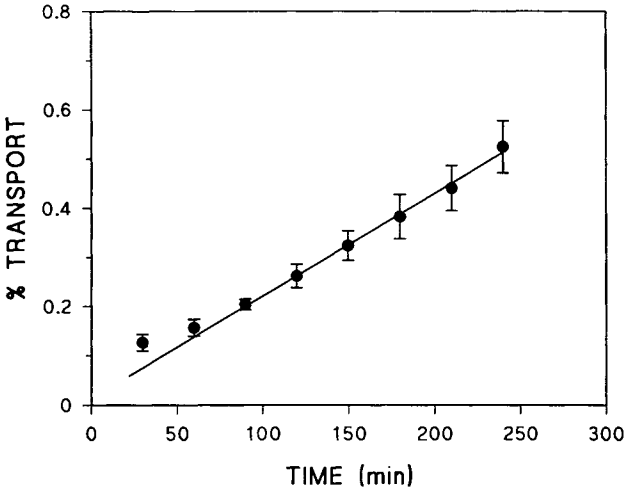

Fig. 1. *In vitro* transport of ^{14}C-polyethylene glycol across CACO-2 cell monolayers. Agitation: 400 rpm, 2.5°. Mean ± SD (n = 6).

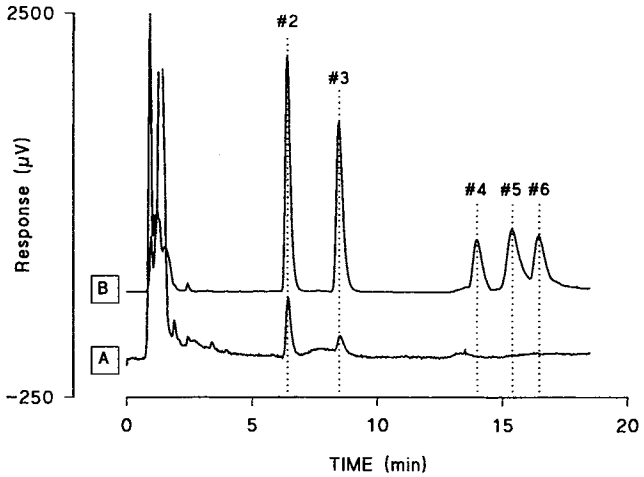

Fig. 2. Chromatograms of (A) a sample taken at time 40 min from the basolateral compartment and (B) authentic samples of testosterone (peak 2), androstenedione (peak 3), etiocholanolone (peak 4), 5-α-androstenedione (peak 5), and androsterone (peak 6). Peak 1 is the solvent front.

course of the experiments. Hence, authentic samples of testosterone and various metabolites were injected into the HPLC system. Figure 2 shows a chromatogram (A) of a sample taken from the basolateral compartment 40 min after the start of the experiment and a chromatogram (B) of testosterone (peak 2), androstenedione (peak 3), etiocholanolone (peak 4), 5-α-androstenedione (peak 5), and androsterone (peak 6). It appears that the unknown peak in the sample chromatogram coelutes with androstenedione. In order to characterize the unknown compound further, the column effluent containing the corresponding peak was isolated and concentrated by evaporation *in vacuo*. Mass spectra were recorded from these samples and from authentic samples of testosterone and various metabolites. The results from the mass spectrometry study are listed in Table II, and they support the hypothesis that the metabolite formed is indeed androstenedione.

These observations indicate the presence of 17-β-hydroxysteroid dehydrogenase in differentiated CACO-2 cells. It is well-known that 17-β-hydroxysteroid dehydrogenase is responsible for the *in vivo* biotransformation of testosterone and estradiol into androstenedione and estrone, respectively (13). The formation of 17-β-hydroxysteroid dehydrogenase by differentiated CACO-2 cells was further supported by the formation of estrone upon incubation of estradiol with CACO-2 cells as determined by HPLC analysis and mass spectrometry (results not shown).

CACO-2 cells have previously been shown to form various enzymes such as alkaline phosphatase (14), protein kinase (15), dipeptidylpeptidase, and sucrase-isomaltase (16,17), but the formation of 17-β-hydroxysteroid dehydrogenase has not been reported before. It is likely that other enzymatic systems will be identified in differentiated CACO-2 cells in the future, emphasizing that the model is not yet fully characterized in terms of metabolism properties. Thus, so far the potential of the CACO-2 transport model for screening transport characteristics of drugs that undergo metabolism is not clear. It is noteworthy that incubation of androstenedione or estrone with CACO-2 cells did not afford any sign of reversible metabolism.

Typical plots of the time course of testosterone and androstenedione found in the basolateral compartment after the addition of 10^{-5} *M* testosterone to the apical compartment are depicted in Fig. 3. With an initial apical concentration of 10^{-5} *M* testosterone, the flux based on androstenedione found in the basolateral compartment is approximately 13% of that measured on the basis of testosterone. Increasing the initial testosterone concentration to 10^{-4} *M*, however, makes this value drop to about 1.7%. This indicates enzymic saturation at substrate concentrations of 10^{-4} *M* or more.

Apparent permeability coefficients (P_{app}) of testosterone transport across the CACO-2 cell monolayers were thus determined from plots similar to Fig. 3, but utilizing an initial concentration of 10^{-4} *M*. At this "high" concentration the formation of androstenedione is relatively small and hence negligible in the calculation of P_{app} for testosterone. Corresponding permeability coefficients for androstenedione were determined from separate transport experiments. The permeability coefficients for testosterone and androstenedione obtained under identical transport conditions (400 rpm and 2.5°) were 66 (±7) * 10^{-6} (n = 26) and 84 (±7) * 10^{-6} (n = 9) cm/sec, respectively. It is evident that androstenedione more readily penetrates the cell monolayer as compared to testosterone (by a factor of approx. 1.3).

The observations that testosterone is metabolized by CACO-2 cells, whereas androstenedione is not, and, further, that the compounds possess different permeability characteristics clearly suggest androstenedione to be superior to testosterone for characterization of the CACO-2 transport model.

As pointed out previously (5–7,18–20) determination and control of the thickness of the unstirred water layer at the interface between the bulk solution and the cell monolayer is of greater importance, especially when studying transport of lipophilic substances. The thickness of this un-

Table II. Liquid Chromatography and Mass Spectrometry Data

Compound	Retention[a]	M_W	m/z[b]	Molecular ion
Testosterone	6.6/5.0	288	124, 288, 147, 256, 203, 133, 135, 165, 228, 210	288
Peak 2[c]	6.6/5.0	?	124, 288, 147, 246, 133, 165, 135, 203, 131, 210	288
Androstenedione	8.1/6.4	286	286, 124, 148, 244, 123, 201, 131, 162, 145, 135	286
Peak 3[c]	8.1/6.4	?	124, 286, 148, 244, 133, 123, 162, 147, 301, 135	286

[a] Retention time (min)/capacity factor.
[b] Ten major EI fragments with m/z larger than 120 listed in order of decreasing relative abundance.
[c] Refer to Fig. 2.

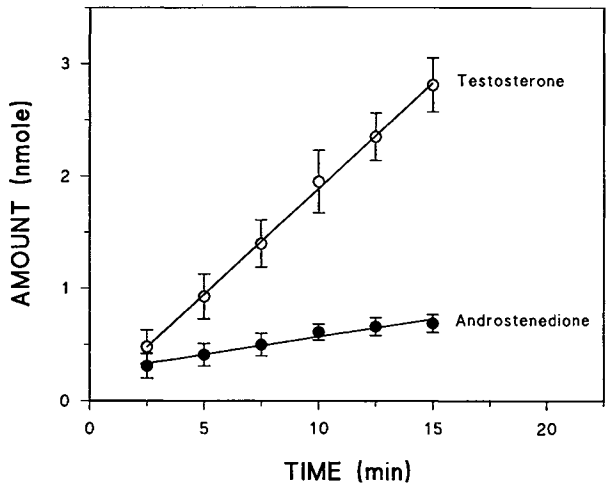

Fig. 3. Time courses of testosterone and androstenedione appearing in the basolateral compartment after addition of 10^{-5} M testosterone to the apical compartment. Mean ± SD (n = 6).

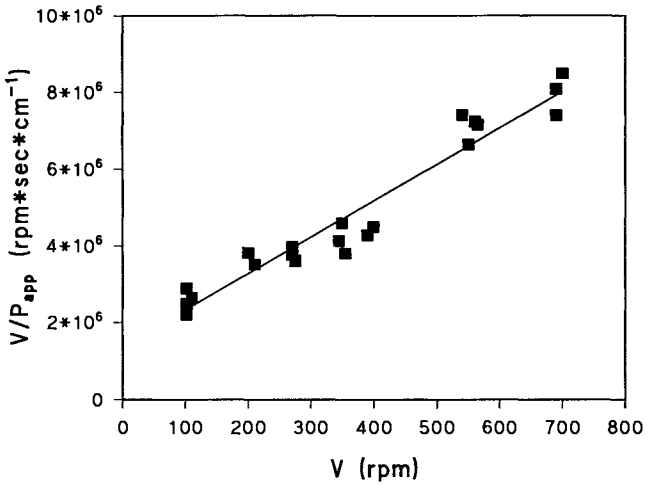

Fig. 5. Linear plot of agitation rate (V) divided by apparent permeability coefficient (P_{app}) as a function of agitation rate (V).

stirred aqueous layer and hence its influence on permeability are controlled by the agitation used in the transport experiment. In our system agitation is controlled by the vibrational speed of the plate shaker and by the angle by which the plate shaker is positioned relative to horizontal. The system allows us to adjust the "stirring" rate from 100 to 1000 rpm and the angle relative to horizontal from 1 to 5°.

In order to determine the thickness of the unstirred water layer at various agitation rates, we have chosen a fixed angle of 2.5°. The method used is similar to that previously described by Karlsson and Artursson (7).

Figure 4 shows representative plots of androstenedione found in the basolateral compartment at different agitation rates, the initial concentration being 10^{-4} M. The flux and hence the permeability coefficient increase with increasing agitation rate, indicating a reduction in the thickness of unstirred water layer with increasing agitation speed.

As shown previously (7,21), the following relationship exists:

$$\frac{V}{P_{app}} = \frac{1}{K} + V\left(\frac{1}{P_c} + \frac{1}{P_f}\right) \quad (2)$$

where V is the agitation rate, K is a constant, and P_c and P_f are the permeabilities across the cell monolayer and the polycarbonate filter, respectively. Accordingly, a plot of V/P_{app} vs V is thought to give a straight line, with the slope being equal to the sum of the $1/P_c$ and $1/P_f$, i.e., the sum of the cell monolayer and the polycarbonate filter resistances (R_c and R_f).

From Fig. 5 the slope is determined by linear regression analysis to 9483 (±204) sec/cm (r = 0.970; n = 24). On the basis of this relationship, the unstirred water layer thickness at various agitation rates can be calculated according to the method described by Artursson and Karlsson (7). This is illustrated in Fig. 6. It is shown that the system is capable of controlling the thickness of the unstirred water layer from about 200 to 1000 μm. At agitation rates higher than about 800 rpm, the cells start to detach from the polycarbonate filter as reflected in a decrease in transepithelial resistance. Our transport model thus makes it possible to simulate the

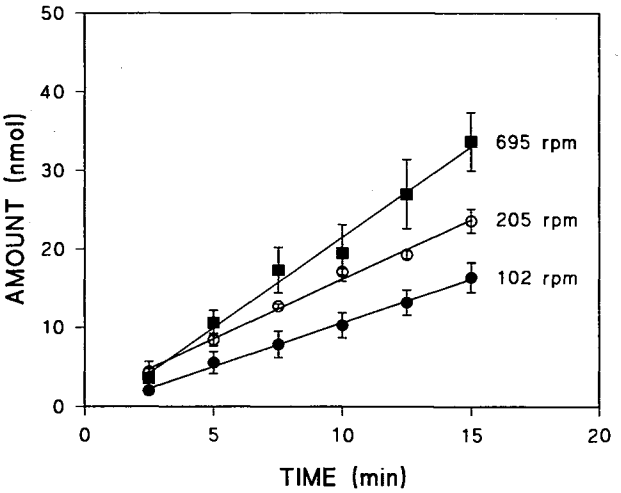

Fig. 4. *In vitro* transport of androstenedione at different agitation rates. Mean ± SD (n = 4).

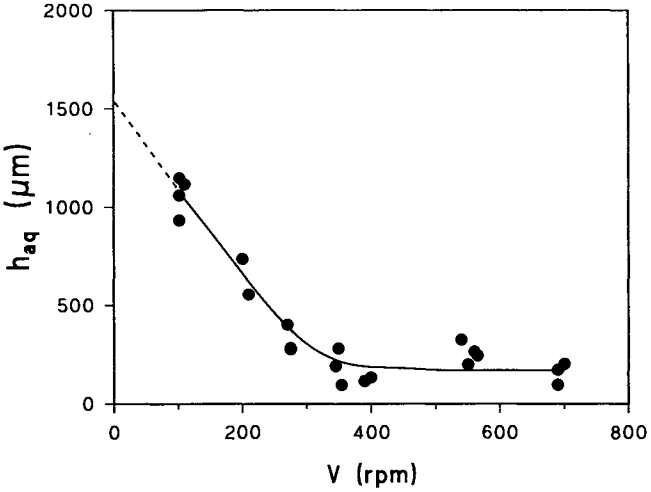

Fig. 6. Thickness of the unstirred water layer as a function of agitation rate. Data points refer to single experiments.

thickness of the unstirred water layer adjacent to the epithelial membrane in the intestine, which has previously been suggested to vary in the range of about 100 to 800 μm in the rat model (19,22).

In conclusion, this study shows that testosterone is metabolized by CACO-2 cells to androstenedione. This indicates the formation of 17-β-hydroxysteroid dehydrogenase by differentiated CACO-2 cells. No reverse metabolism was observed. Androstenedione is thus considered superior to testosterone for determination of the unstirred water layer thickness in the CACO-2 transport model. The metabolization process is significant at low initial testosterone concentrations, i.e, at 10^{-5} *M* testosterone the flux of androstenedione is about 13% that of testosterone. This, together with the different permeability characteristics of testosterone and androstenedione (a factor of approx. 1.3 in favor of androstenedione), emphasizes that determination of the unstirred water layer thickness without taking metabolism into account is incorrect. At higher initial concentrations, however, the relative amount of androstenedione formed is small. Thus, at an initial concentration of testosterone of 10^{-4} *M*, the flux based on androstenedione is only about 1.7% of that based on testosterone, indicating saturation of the metabolization process and, further, that metabolization is negligible as far as determination of the water layer thickness is concerned.

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