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# CHOLESTATIC STEROID HORMONES INHIBIT TAUROCHOLATE UPTAKE INTO ISOLATED RAT HEPATOCYTES

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**Abstract**—The effect of three cholestatic steroids (norethandrolone, 17-β-estradiol and progesterone) on hepatocellular uptake and secretion of taurocholate was studied in isolated rat liver cells. The steroids decreased the rate of taurocholate uptake. Norethandrolone inhibited uptake noncompetitively with a $K_i$ of 18 μM, but had no effect on the activation energy of uptake. 17-β-estradiol and progesterone reduced taurocholate uptake by 50 per cent at concentrations between 40 μM and 50 μM. The secretion of taurocholate from taurocholate-loaded cells was slightly increased by all three steroids at concentrations below 100 μM. A 60 per cent inhibition of secretion was observed in the presence of 500 μM norethandrolone. Interference of cholestatic steroids with hepatocellular bile acid uptake may be an important step in the pathogenesis of intrahepatic cholestasis.

A variety of drugs [1] cause intrahepatic cholestasis, which, like any form of cholestasis, is characterized by reduced bile flow and high concentrations of bile acids in the liver and in the serum. The primary event of drug-induced cholestasis is still unknown. Several different mechanisms have been implicated in the pathogenesis of this disease, such as altered bile acid synthesis [2, 3], precipitation of lithocholate or drugs in the canaliculi or pericanalicular space [4, 5], alterations of the canalicular membrane [6] and of the microfilaments [7].

In the course of enterohepatic circulation each bile acid molecule passes through the liver about 20 times before being finally eliminated [8, 9]. Interference with the transport rate of either uptake from the sinusoidal side or secretion into the canaliculi could therefore lead to reduced bile flow and accumulation of bile acids.

To study whether cholestatic steroids interfere with bile acid transport, isolated rat liver cells were used. Isolated hepatocytes allow independent measurements of uptake and secretion; we have recently shown that both processes are carrier-mediated, energy-dependent, and saturable [10, 11].

Three steroid hormones of different cholestatic potencies were tested for their interference with taurocholate transport: Norethandrolone, an anabolic drug which causes jaundice [12], 17-β-estradiol, which may be responsible for cholestasis during pregnancy, and progesterone, of which involvement in cholestasis is uncertain [13].

Taurocholate is the major bile acid in the rat, and was therefore used in the experiments. This bile acid has the additional advantage of passing through the liver cell virtually unmetabolized.

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## MATERIALS AND METHODS

*Materials.* Tauro[*carbonyl*-$^{14}$C]cholic acid, 50 mCi/m-mole was from Radiochemical Center (Amersham, England), [*carboxyl*-$^{14}$C]dextran, molecular weight 75000, 1.3 mCi/g from NEN Chemicals (Dreieichenhain, Germany). Collagenase grad 2 (from Clostridium histolyticum) and antimycin A were delivered by Boehringer (Mannheim, Germany). Hyaluronidase type 1 (from bovine testes) was purchased from Sigma (St Louis, U.S.A.), taurocholic acid and trypan blue were from Serva (Heidelberg, Germany). Carbonylcyanide-*m*-chlorophenylhydrazone was delivered from Calbiochem (Los Angeles, U.S.A.). Silicone oils, types AR 200 and AR 20, were from Wacker Chemie (München, Germany). Norethandrolone (17α-ethyl-17β-hydroxyestr-4-en-3-on) was a gift from Searl (Chicago, U.S.A.). 17-β-Estradiol, progesterone and all other chemicals were purchased from Merck (Darmstadt, Germany) at the highest purity available.

*Isolation of rat liver cells.* Hepatocytes were prepared from male Sprague–Dawley rats (300 g) (Tierzuchtanstalt Ivanovas, Kisslegg). Standard laboratory diet and water were given *ad lib.*

Isolated liver cells were prepared according to the method of Berry and Friend [14], as modified by Seglen [15] and Baur [16]. A cannula was inserted into the portal vein under ether anesthesia, and perfusion was started with $Ca^{2+}$-free Hanks medium at 37°.

After excision of the liver, perfusion was continued with a recirculating medium supplemented with 0.5 mM $Ca^{2+}$, 0.1% hyaluronidase and 0.05% collagenase. After 10–15 min the portal vein became leaky, and perfusion was continued via the vena cava. After

another 30 min the liver was minced and poured in a round-bottomed flask, where dissociation of cells was allowed to continue by slow rotation for 15 min. During the entire procedure the medium was aerated with carbogen. After washing, the cells were stored at 0° as a suspension (about 40 mg cell protein/ml) in a standard medium which consisted of 137 mM NaCl, 5.2 mM KCL, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 5 mM glucose, buffered with 3 mM phosphate and 10 mM Tris to pH 7.3.

*Viability of liver cells.* Viability of the cell preparations was tested according to the criteria recently published [16, 17], i.e. trypan blue staining (less than 8%), stimulation of oxygen consumption by succinate (less than 20%) and the respiratory control ratio (at least 1.9).

*Transport of taurocholate.* To measure uptake of taurocholate, aliquots of the cell suspension were diluted with the standard medium to a final concentration of about 2 mg cellular protein/ml. After 5 min preincubation at 37°, 10 μl of 60 nCi [$^{14}$C]taurocholate were added to 1 ml suspension and various concentrations of unlabeled taurocholate. The cell suspensions were slowly stirrred throughout the experiments.

To measure secretion of taurocholate, cells (6–7 mg cellular protein/500 μl) were incubated with 90 nCi [$^{14}$C]taurocholate and various amounts of unlabeled taurocholate. The oxygen supply was maintained by streaming oxygen over the surface of the cell suspension. After 15 min, 100 μl of the cell suspension ("loaded cells") were transferred to 2 ml of medium, free from bile acids.

In the uptake as well as in the secretion studies norethandrolone, progesterone and 17-β-estradiol were dissolved in ethanol and added in all experiments in a volume of 10 μl to the cell suspensions. 10 μl ethanol alone did not affect taurocholate transport.

*Termination of transport reactions.* Both uptake and efflux reactions were stopped by separating the cells from the medium by centrifugal filtration as described earlier [8, 10]. 200 μl samples of the cell suspension were placed in centrifuge tubes which contained at the bottom 50 μl 3 M KOH covered by 100 μl silicone oil. The density of this silicone layer is higher than that of the medium and lower than that of the 3 M KOH. On centrifugation the cells pass through the silicone and are denatured in the KOH layer. Initial rates of uptake were obtained from measurements at 15, 30, 45 and 60 sec after the addition of taurocholate. Initial rates of secretion were obtained from the values 15, 30, 45, 60, 75, 90, 120 and 180 sec after transferring the "loaded" cells into a medium free of bile acids. Radioactivity in the cell precipitate and the supernatant was analysed in Bray's solution in a Berthold liquid scintillation counter.

*Special analytical methods.* Cellular protein was determined by a modified biuret method [19]. For the trypan blue exclusion test, equal volumes of the cell suspension and of a trypan blue solution (0.4% w/v in the incubation medium) were mixed. Oxygen consumption was measured with a Clark electrode at pH 7.3 and 37°.

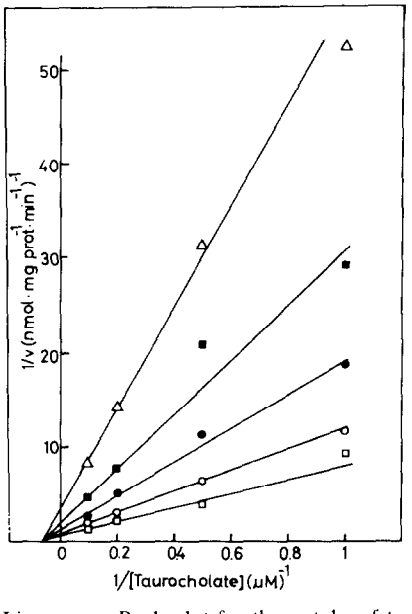


Fig. 1. Lineweaver–Burk plot for the uptake of taurocholate showing inhibition by norethandrolone. Liver cells (2 mg cellular protein) from the stock suspension were preincubated at 37° in 1 ml standard medium at pH 7.3 for 5 min. Norethandrolone was added to the cell suspension 10 sec before the addition of taurocholate. 200 μl samples were withdrawn and immediately centrifuged at the times described in the Methods section. Uptake of taurocholate was linear for 1 min. From the slope of the straight lines the initial velocity of uptake was calculated. Concentrations of norethandrolone: 0 μM (□), 10 μM (○), 25 μM (●), 50 μM (■), 100 μM (△).

## RESULTS

*Inhibition of taurocholate uptake.* The anabolic drug norethandrolone, which causes cholestasis *in vivo* [12, 20], inhibits uptake of taurocholate by isolated liver cells (Fig. 1). The inhibition is noncompetitive, as demonstrated by the common intercept on the abscissa in the Lineweaver–Burk plot. To determine the inhibitor constant the reciprocal velocity was plotted versus concentration of the inhibitor according to Dixon (Fig. 2). The inhibitor constant ($K_i$) for norethandrolone is 18 μM.

In these experiments norethandrolone was added to cell suspensions 10 sec before the addition of the bile acid. To test whether the length of preincubation with norethandrolone alters the inhibitory effect, cells were preincubated with 25 μM norethandrolone for 20 min prior to taurocholate addition. This extension of the preincubation period did not increase the inhibition of uptake.

In Fig. 3 the temperature dependency of taurocholate uptake is plotted according to Arrhenius. The parallel shifting of the lower line indicates that the velocity of uptake is reduced in the presence of norethandrolone. Norethandrolone did not alter the value for the activation energy of uptake (about 28 kcal/mole) which was calculated from the slopes of the straight lines.

Taurocholate uptake is also inhibited by 17-β-estradiol and progesterone. Both drugs cause a 50 per cent inhibition at 40 to 50 μM (Table 1) at an extracellular taurocholate concentration of 10 μM.

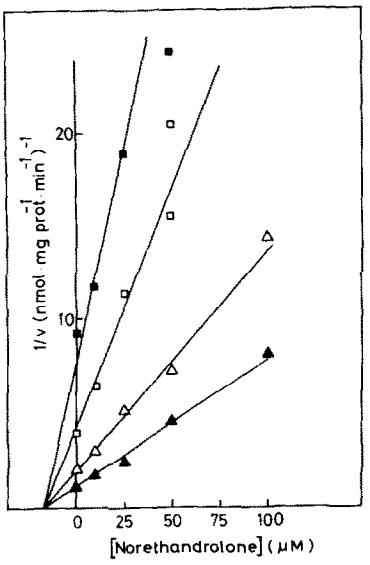


Fig. 2. Dixon plot for inhibition of taurocholate uptake by norethandrolone. The reciprocal velocities of Fig. 1 were plotted against the concentrations of norethandrolone. Concentrations of taurocholate: 1 μM (■), 2 μM (□), 5 μM (△), 10 μM (▲).

*Effect of cholestatic compounds on secretion.* When cells are loaded with various amounts of taurocholate and then transferred to a medium free of bile acid, taurocholate is released at a constant rate for 2–3 min [11].

The effects of norethandrolone on secretion of taurocholate are complex. At low concentrations (5–10 μM), the rate of excretion is not decreased, but shows a small increase of about 10 to 15 per cent. At the much higher concentration of 500 μM norethandrolone strongly inhibits secretion (Table 2, Fig. 4).

17-β-estradiol and progesterone similarily to norethandrolone slightly increased secretion at low concentrations up to 100 μM. Higher concentrations than 100 μM were not used on account of the low solubility of these two steroids.

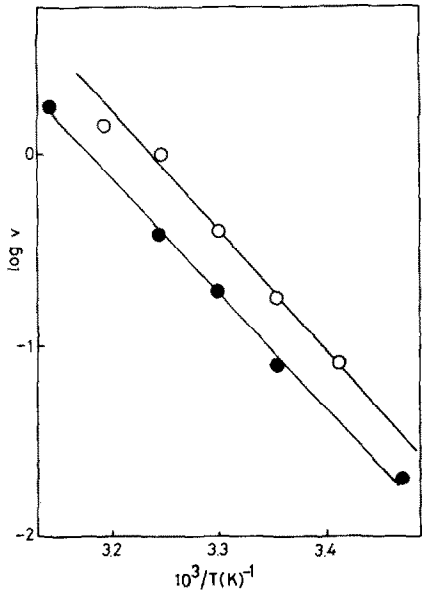


Fig. 3. Arrhenius plot: effect of temperature on taurocholate uptake after the addition of norethandrolone. Norethandrolone (50 μM) was added 10 sec before taurocholate (10 μM) to the cell suspension at various temperatures. Initial rates of uptake were measured as described in Fig. 1. $V$ is expressed in nmoles/mg cellular protein × min and plotted against $1/T$. (○) without norethandrolone, (●) with norethandrolone. Calculations based on the slopes of the straight lines indicate an activation energy of about 28 kcal/mole.

Table 1. Inhibition of taurocholate uptake by steroid hormones

| Steroid hormone | $I_{50}$-value (μM) |
|---|---|
| Norethandrolone | 18 ∓ 4 |
| 17-β-Estradiol | 48 ∓ 7 |
| Progesterone | 42 ∓ 3 |

* The initial rates of uptake were measured as described in the legend to Fig. 1. The steroids were added to the cell suspension (2.2 mg protein/ml) 10 sec before the addition of taurocholate. The data represent the steroid concentrations at which 50 per cent inhibition is observed and are the means of 4 measurements. The taurocholate concentration was 10 μM.

## DISCUSSION

Norethandrolone, 17-β-estradiol and progesterone inhibited taurocholate uptake into isolated liver cells (Table 1). This impairment of transport does not seem to require metabolism of the steroids, because they all exerted their effect immediately and, as shown for norethandrolone-inhibition, independently of the duration of the preincubation period. Direct interference of the steroids with the active site of the carrier protein appears unlikely in view of the noncompetitive type of inhibition which has been shown for norethandrolone (Fig. 1). The inhibition may rather be due to the capacity of the lipophilic steroids to enter the plasma membrane and to thereby affect the motility of the fatty acid chains [21]. Such changes in the lipid environment have been shown to alter the activity of membrane carriers [22].

The daily dosage of norethandrolone in man is 10–30 mg [23]; assuming uniform distribution

Table 2. Effect of steroid hormones on secretion of taurocholate*

| Steroid hormone | Steroid concentration (μM) 5 | 20 | 50 | 100 | 500 |
|---|---|---|---|---|---|
| Norethandrolone | 101 | 107 | 115 | 82 | 41 |
| 17-β-Estradiol | 98 | 113 | 119 | 117 | — |
| Progesterone | 104 | 114 | 111 | 90 | — |

* Cells were loaded with 10 μM taurocholate for 15 min. The steroid hormones were dissolved in 10 μl ethanol and were added to the cellular suspension (0.3 mg protein/ml) immediately after the 20-fold dilution of the cells. Ethanol (10 μl) alone did not affect secretion of taurocholate. The data represent the rates of secretion during the first 3 min as per cent of control at various steroid concentrations and are the mean of duplicate measurements from two cell preparations. 17-β-estradiol and progesterone are insoluble at 500 μM.

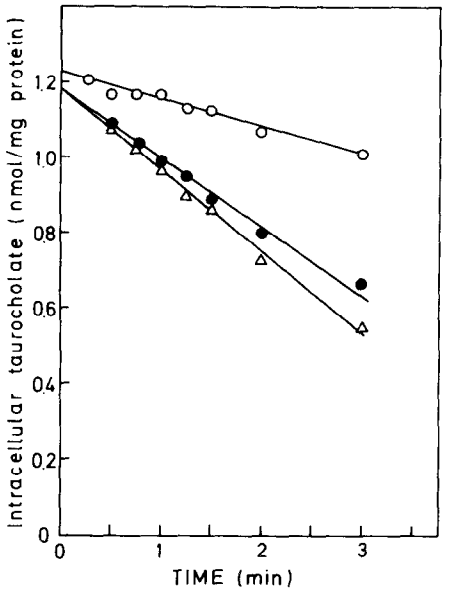


Fig. 4. Influence of norethandrolone on secretion of taurocholate. Liver cells (6 mg cellular protein) were preincubated at 37° in 500 μl standard medium for 3 min. The cells were subsequently "loaded" with 10 μM taurocholate for 15 min. To measure the secretion of taurocholate, 100 μl of the cell suspension were transferred to 2 ml standard medium free of bile acids. Norethandrolone dissolved in 10 μl ethanol was added to the cell suspensions at a final concentration of: 0 μM (●), 50 μM (△), 500 μM (○). Samples of 200 μl were withdrawn and immediately centrifuged at the times described in the Methods section.

throughout the body, the concentration would reach approximately 2 μM. This represents about one-tenth of the concentration required for 50 per cent inhibition *in vitro*. Even during pregnancy serum concentrations of 17-β-estradiol and progesterone may approach 1 μM only. However, simultaneous interactions of several endogenous steroids and drugs at the membrane *in vivo* must be considered. Therefore, levels of single steroids added *in vitro* can hardly be compared with those found *in vivo*, which add to the sum of other steroids already being present.

In contrast to the hepatocellular uptake of taurocholate secretion has been little affected by the steroids. The $V_{max}$ of taurocholate secretion in the absence of steroids [11] corresponds to the $T_m$-value both *in vivo* and in the isolated perfused liver [24, 25]. This strongly suggests that isolated hepatocytes used in the present study maintain their physiological function to secrete bile acids. Intactness of the plasma membrane of enzymatically prepared hepatocytes is also indicated by the presence of glucagon, epinephrine, and insulin receptors on the surface of the free cells [26]. Furthermore Wisher and Evans could show that the uneven distribution of hydrolytic surface enzymes at the different faces of the plasma membrane, i.e. the blood sinusoidal, the bile canalicular and the contiguous (lateral) face is still preserved in isolated liver cells [27]. The small increase of the secretion rates in the presence of low steroid concentrations may be ascribed to inhibition of reuptake of previously secreted taurocholate. Secretory inhibition in the presence of very high norethandrolone concentrations (500 μM) may be due either to interference with the plasma membrane or to the known inhibition of the mitochondrial respiratory chain by steroids [28, 29]. Considering that intrahepatic cholestasis is caused by much lower steroid concentrations *in vivo*, it is doubtful whether the effect of 500 μM norethandrolone *in vitro* has any relevance to the genesis of intrahepatic cholestasis.

Inhibition of bile acid uptake *in vivo* could promote intrahepatic cholestasis in the following way. Inhibition of bile acid uptake by cholestatic steroids impairs transcellular bile acid transport from blood into bile [30]. Consequently, the intracellular bile acid concentration decreases. Since bile acid synthesis is regulated by a feedback mechanism, a lowered intrahepatic bile acid concentration releases product inhibition of bile acid formation, thus increasing bile acid synthesis [31]. Quantitatively, *de novo* synthesis may be insignificant, since it restores only a small portion of the bile flow. However, qualitative aspects may be important, since steroid hormones shift the pattern of bile acid synthesis to the production of hepatotoxic mono- and dihydroxylated bile acids by inhibition of microsomal hydroxylation reactions [3, 32, 33]. Such hepatotoxic bile acids may change the properties of the canalicular membrane, and lead to failure of bile acid secretion. This has been recently reported in the case of lithocholate [34].

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