Uptake of [^{3}H]bilirubin in freshly isolated rat hepatocytes: role of free bilirubin concentration

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Abstract Hepatocytic transport of physiological concentrations of unconjugated bilirubin (UCB) has not been determined in isolated liver cells. Initial uptake of highly purified [^{3}H]UCB was measured in rat hepatocytes in the presence of human serum albumin at various free, unbound UCB concentrations, [UCB]. At [UCB] = 42 nM (below aqueous solubility of 70 nM), uptake was strictly temperature dependent; this was much less evident at [UCB] = 166 nM (supersaturated). At low, physiological UCB concentrations, specific UCB uptake showed saturative kinetics with an apparent K_m of 41 nM, indicating carrier-mediated transport. With aqueous supersaturation, UCB entered hepatocytes mainly by passive diffusion.

Key words: Unconjugated bilirubin; Hepatocyte; Saturation

1. Introduction

The transport mechanisms of different organic anions across the membrane of liver cells have been investigated extensively [1–3]. The endogenous organic anion, unconjugated bilirubin (UCB), the end product of heme catabolism, is taken up by the liver, which secretes it into the bile after metabolic biotransformation (conjugation) [4]. The uptake of UCB across the basolateral membrane of the hepatocyte has been regarded as a carrier-mediated process on the basis of experiments performed with rat liver plasma membrane vesicles enriched in the basolateral portion [5]. More recently, it has been proposed that the passage of UCB across membranes may occur by spontaneous diffusion [6,7], thus challenging the concept that specific carrier protein(s) accounts for this transport function. Though studies on UCB transport have been performed in the whole animal [8,9], the tumoral Hep G2 cell line [10] and liver plasma membrane vesicles [5], most of these were done at high, non-physiological UCB concentrations and little data have been obtained in the more physiological model of isolated hepatocytes. The present study was undertaken to investigate UCB uptake by freshly isolated rat hepatocytes at substrate concentrations both below and above the aqueous solubility limit for unbound (free) UCB, in the presence of the physiological UCB-binding protein of plasma, human serum albumin (HSA).

2. Materials and methods

2.1. Drugs and chemicals

Ethylene glycol-bis(β-aminoethyl ether *N,N,N′,N′*-tetraacetic acid (EGTA), bovine serum albumin (BSA), HSA (fatty acid free, lot. no. 93H9345), sodium ascorbate, collagenase type IV (C-5138) and unlabelled bilirubin (UCB) were purchased from Sigma Chemical (St. Louis, MO, USA). Bilirubin was purified according to McDonagh and Assisi [11].

[^{3}H]Bilirubin was labelled biosynthetically in bile-fistula rats by intravenous infusion of 3,5-[^{3}H]δ-aminolevulinic acid, as described by Lester and Klein [12], and then purified from the bile as described by Ostrow et al. [13]. The specific activity was 30 000 dpm/μg. All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

2.2. Isolation of rat hepatocytes

Adult female Wistar rats weighing 150–200 g were used in all experiments. They were fed ad libitum and received humane care according to international regulations. The local Animal Care Committee approved the protocol.

Rats were anesthetized with sodium thiopental (70 mg/kg body weight, intraperitoneal) and hepatocytes isolated by collagenase perfusion, as described by Seglen [14] and modified by us [15]. Briefly, livers were perfused 'in situ' for 5 min via the portal vein with 100 ml of a modified Hank's balanced salt solution (HBSS) supplemented with 5 mM Tris and 0.5 mM EGTA, pH 7.40 at 37°C. The perfusate was oxygenated by passing through oxygen-permeable tubing (Silicone Tubing, Baxter Healthcare Corporation, Irvine, CA, USA) inside an appropriate glass container with 95% O_2 and 5% CO_2, at a pressure of 80 mm Hg. Air bubbles were avoided by connecting a disposable Nylon filter in line between the oxygenator and the inflow. After the initial flushout, the perfusion was followed in a recirculating buffer system with 150 ml of HBSS in the absence of EGTA, supplemented with 1.2 mM $CaCl_2$, 0.025% collagenase type IV and 1% BSA. The cell suspension was centrifuged ($50 \times g$ for 3 min) and the pellet was resuspended two times in HBSS solution containing 1% BSA and 1.2 mM $CaCl_2$. Only viable cells, which excluded greater than 85% of 0.4% trypan blue dye, were utilized for the experiments.

2.3. Measurement of UCB uptake by hepatocytes

Freshly isolated, washed hepatocytes were suspended in HBSS in the absence of albumin, supplemented with 1.2 mM $CaCl_2$. A constant concentration of [^{3}H]UCB (0.01 μCi/ml) plus varied amounts of unlabelled UCB were dissolved in 30 μl DMSO and diluted with 3.0 ml of HBSS solution containing 200 μM HSA and 2 mM sodium ascorbate, pH 7.4. The uptake of UCB was measured by the addition of 0.5 ml of the UCB-HSA solution to the hepatocytes (2×10^6 cells in 0.5 ml) maintained at 37°C. The free, unbound UCB concentration,

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[UCB], in the medium ranged from 8 to 210 nM, calculated using the affinity constants (K'_f) of HSA for UCB previously described in detail by Pascolo et al. [5].

After the chosen interval of incubation, the cell suspension was centrifuged (13 000×g for 10 s) and the pellet washed twice with 1 ml of cold PBS and centrifuged (13 000×g per 10 s). After each spin, the supernatant was removed by aspiration through an 18 G needle connected to a vacuum pump. Radioactivity in the pellet was determined by dissolving the cells in 0.3 ml of Solvable (NEF-910) (Dupont NEN, Boston, MA, USA), of which 50 μl was mixed with 2 ml of liquid scintillation cocktail (Optiphase Hisafe 3, Wallac, Finland), for radioassay in an LKB Wallac liquid scintillation system. Counts were corrected for quenching using external standards. In preliminary experiments, the time course of UCB uptake was determined at two different [UCB] (42 and 166 nM), after incubations for 15, 30, 45 s and 1, 2, 5, 10, 15, 30, 60, 90 min. Uptake was linear up to 60 s. Accordingly, the 1 min sampling time was chosen to determine the initial uptake rate.

According to what described previously in liver plasma membrane vesicles [5,16], non-specific binding of UCB to hepatocytes was determined from a paired measurement of UCB uptake by the cells incubated at 4°C. The difference between the 37 and 4°C values was considered as specific uptake of UCB. For each determination, UCB transport was measured in duplicate in at least three separate cell preparations.

2.4. Statistical analysis

Results are expressed as mean ± S.D. of at least three experiments. Kinetic analyses (K_m and V_{max}) were determined from a computer-assisted program (SigmaPlot, Landel Scientific, San Rafael, CA, USA) according to the Michaelis–Menten equation.

3. Results

Fig. 1 shows the time course of UCB uptake measured at a [UCB] of 42 nM (undersaturated) [17], both at 37 and 4°C over a period of 90 min. Cumulative uptake at 4°C increased linearly with time (uptake rate constant). At 37°C, by contrast, the uptake rate was initially much faster and declined progressively, so that cumulative UCB uptake reached a virtual plateau from 30 min onwards.

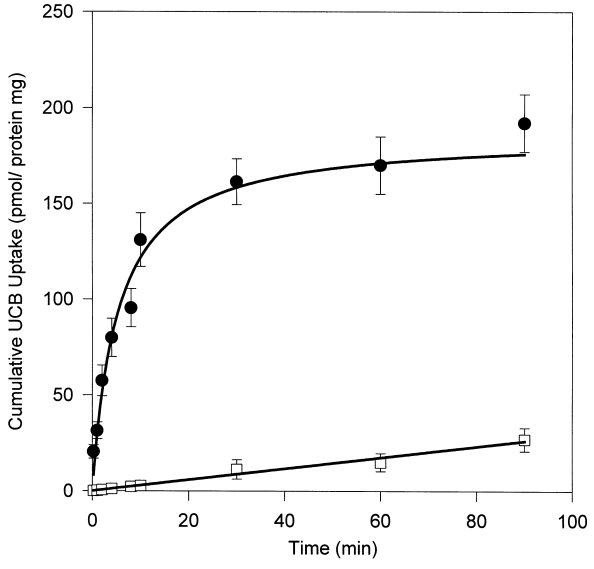

Fig. 1. Time course of [^{3}H]UCB uptake in isolated rat hepatocytes measured at a free UCB concentration [UCB] of 42 nM, in the presence of 100 μM HSA and 2 mM ascorbate at pH 7.4. Uptake was measured as described in Section 2 either at 37 (●) or at 4°C (□). Each point represents the mean ± S.D. of duplicate determinations performed with at least three different hepatocyte preparations. Uptake at 4°C was linear ($y = 0.29x$, $r^2 = 0.981$), while that measured at 37°C was curvilinear and reached a virtual plateau from 30 min onwards.

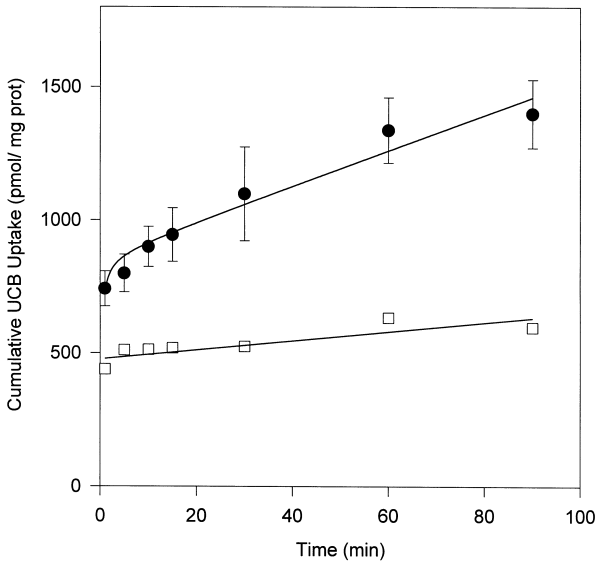

Fig. 2. Time course of [^{3}H]UCB uptake in isolated rat hepatocytes measured at a free UCB concentration [UCB] of 166 nM. Uptake was measured as described in Section 2 either at 37 (●) or at 4°C (□). Each point represents the mean ± S.D. of duplicate determinations performed with at least three different hepatocyte preparations. Note that the ordinate scale is about 10-fold that in Fig. 1. Uptake at 4°C was linear ($y = 476+1.72x$, $r^2 = 0.992$) while that measured at 37°C increased slightly up to 10 min and then increased linearly ($y = 800+6.3x$, $r^2 = 0.979$).

When uptake was assessed at a [UCB] of 166 nM (2.4× aqueous saturation [17]) (Fig. 2), uptake at 4°C was likewise linear, but the uptake velocity, as indicated by the slope of the line, was almost six times that found at a [UCB] of 42 nM (1.7 vs. 0.29). The uptake at 37°C was curvilinear, but the increments with time were much less marked than under unsaturated conditions. In addition, the uptake plots at 4°C and 37°C intercepted the y axis at respective values near 500 and 750 pmol UCB/mg protein/min, compatible with immediate precipitation of some of the excess pigment on the cellular surface.

When the linear component at 4°C was subtracted from the corresponding 37°C value and the resultant specific uptake plotted vs. [UCB] (Fig. 3), a clear saturative function was obtained with a K_m of 41 ± 11 nM and V_{max} of 108 ± 8 pmol/mg protein/min.

4. Discussion

In spite of the fact that UCB is one of the most important endogenous cholephilic organic anions in plasma, there are few reported studies of UCB transport by isolated hepatocytes. Iga et al. [18] suggested that UCB uptake by cultured rat hepatocytes was not saturable and concluded that the process was due to passive diffusion. These data were severely flawed due to the absence of albumin in the incubation medium as a reservoir of bound UCB to prevent substrate depletion and by the marked supersaturation of [UCB][17]. More recently, Zucker et al. provided evidence that the spontaneous transmembrane diffusion of UCB across membranes

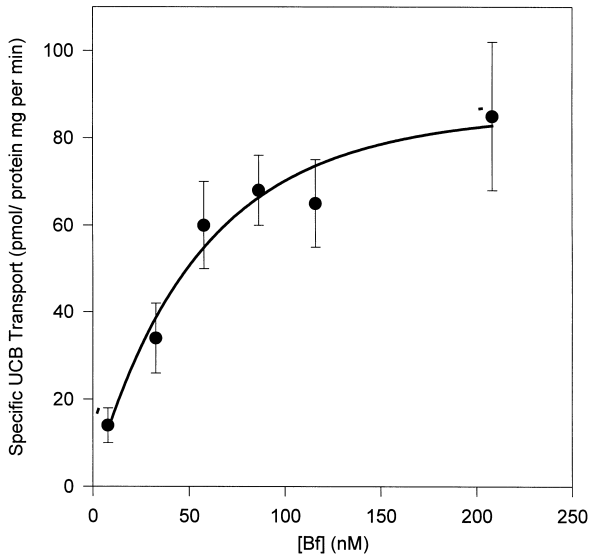

Fig. 3. Saturative behavior of the specific initial uptake of [^{3}H]UCB, calculated by subtraction of the 4°C from the 37°C value at each free bilirubin concentration [UCB]. Each point represents the mean ± S.D. of duplicate determinations performed with at least three different hepatocyte preparations. Calculated $K_m = 41 \pm 11$ nM and $V_{max} = 108 \pm 8$ pmol/mg protein/min.

is rapid and efficient and that the overall transfer is mainly determined by the dissociation rate of UCB from albumin [6]. These data have led these authors to question the need for specific transporting proteins for UCB at the basolateral domain of the hepatocyte. The need for specific transporting systems has also been questioned since, in spite of extensive investigation [3,19], no UCB transporter(s) have been cloned to date.

The data presented indicate that the uptake of UCB by freshly isolated rat hepatocytes is mediated by two different mechanisms, whose relative contributions differ according to the substrate concentration ([UCB]). When the concentration of UCB is well below supersaturation (Fig. 1), the uptake rate is marginal at 4°C, while at 37°C, it is about 50 times greater during the first 10 min. By contrast, when transport is measured at [UCB] well above saturation, the uptake rate at 37°C is less than 2.5 times that at 4°C, despite substantial internalization of UCB into the hepatocytes, and there is extensive, immediate precipitation of UCB onto the cell surfaces [17]. This non-specific interaction of UCB with the cellular surface is thus minimal at low [UCB], while it becomes dominant when [UCB] exceeds saturation. More interesting was the observation that the specific uptake, as indicated by the difference between the cumulative uptake at 37°C minus 4°C (Fig. 3), follows a saturative curve with kinetic constants similar to those reported in rat liver plasma membrane vesicles [5]. This suggests that, under these conditions, UCB is taken up by the hepatocyte mainly by specific, carrier-mediated, saturative mechanism(s), with an apparent affinity (K_m) of the specific uptake processes for UCB in the normal range of free UCB (5–20 mM) concentrations in the intact animal [17].

Our findings indicate that, at high [UCB], non-saturative mechanisms account for more than 45–50% of UCB uptake by the liver cell, but this does not validate the suggestion that specific systems for UCB transport are not needed. Thus, when [UCB] is in the physiological range below aqueous saturation, diffusional UCB uptake at 4°C comprises less than 20% of the total UCB uptake at 37°C. Clearly, both mechanisms (saturative or diffusional) may be operative and the free concentration of UCB seems to determine the relative role of each of them. These data further indicate a need to determine HSA-binding and transport of UCB under experimental conditions where the exact concentration of free substrate [UCB] is calculated and is the physiological range below aqueous solubility.

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