Albumin binding of unconjugated [³H]bilirubin and its uptake by rat liver basolateral plasma membrane vesicles

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Using highly purified unconjugated [³H]bilirubin (UCB), we measured UCB binding to delipidated human serum albumin (HSA) and its uptake by basolateral rat liver plasma membrane vesicles, in both the absence and presence of an inside-positive membrane potential. Free UCB concentrations ($[B_f]$) were calculated from UCB–HSA affinity constants (K'_f), determined by five cycles of ultrafiltration through a Centricon-10 device (Amicon) of the same solutions used in the uptake studies. At HSA concentrations from 12 to 380 μ M, K'_f (litre/mol) was inversely related to $[HSA]$, irrespective of the $[B_t]/[HSA]$ ratio. K'_f was $2.066 \times 10^6 + (3.258 \times 10^8/[HSA])$. When 50 mM KCl was iso-osmotically substituted for sucrose, the K'_f value was significantly lower $\{2.077 \times 10^6 + (1.099 \times 10^8/[HSA])\}$. The transport occurred into an osmotic-sensitive space. Below saturation ($[B_t] \leq 65$ nM), both electroneutral and electrogenic components followed saturation kinetics with respect to $[B_t]$, with K_m values of

28 ± 7 and 57 ± 8 nM respectively (mean $\pm$ S.D., $n = 3$, $P < 0.001$). The V_{max} was greater for the electrogenic than for the electroneutral component (112 ± 12 versus 45 ± 4 pmol of UCB $\cdot$ mg⁻¹ of protein $\cdot$ 15 s⁻¹, $P < 0.001$). Sulphobromophthalein *trans*-stimulated both electrogenic (61%) and electroneutral (72%) UCB uptake. These data indicate that: (a) as $[HSA]$ increases, K'_f decreases, thus increasing the concentration of free UCB. This may account for much of the enhanced hepatocytic uptake of organic anions observed with increasing $[HSA]$. (b) UCB is taken up at the basolateral membrane of the hepatocyte by two systems with K_m values within the range of physiological free UCB levels in plasma. The electrogenic component shows a lower affinity and a higher capacity than the electroneutral component. (c) It is important to calculate the actual $[B_f]$ using a K'_f value determined under the same experimental conditions (medium and $[HSA]$) used for the uptake studies.

INTRODUCTION

Four basolateral membrane transporters have been described that apparently mediate hepatocytic uptake of organic anions (OAs) [1–4]. Some of these transport systems are sensitive to membrane potential (electrogenic) whereas others are not (electroneutral) [5]. As inferred from competitive studies [5,6] these carrier systems are shared by many OAs, including unconjugated bilirubin (UCB), Indocyanine Green, sulphobromophthalein (BSP), iminodiacetic acids and some bile acids.

Though UCB is the natural substrate for these transporters, its uptake has seldom been studied due to instability [7], low aqueous solubility [8,9] and difficulties in measurement of very low UCB concentrations [7,9]. Hepatic transport has, therefore, been characterized mainly by studies utilizing BSP as the OA; the results were then extrapolated to UCB. The findings have suggested that the hepatocytic uptake of OAs is related to both the unbound (free) concentration of the OA and the total concentration of human serum albumin (HSA) [5,10,11]. Extrapolation of these results to UCB has not, however, been validated, and studies are needed with UCB itself. The few studies with UCB have usually been performed with metastable solutions that are supersaturated with unbound UCB, or run at non-physiological high pH values at which UCB solubility is higher

[8]. Precipitation of excess unbound UCB diacid in these systems may have resulted in misinterpretation of data obtained.

In most previous studies, moreover, the unbound/free concentration of UCB ($[B_f]$) has generally been calculated from the accepted affinity constant (K'_f) of UCB for HSA of approximately 6×10^7 litre/mol [12], derived by selective peroxidation of unbound UCB [13]. However, this accepted K'_f was not determined with the same batch of HSA, or in media of the same composition as those in the transport studies, factors that might affect both UCB binding to HSA and the uptake values.

We have recently developed methods to prepare highly labelled [³H]UCB that contains less than 0.01% of label in polar derivatives of UCB [14]. When dissolved in DMSO and then added to aqueous systems at pH 7.0 under argon or nitrogen, this preparation is stable for up to 1 h, particularly if albumin is present. This permits studies of [³H]UCB metabolism, physical chemistry and transport at near physiological pH and at $[B_t]$ values below saturation (65 nM) [9] if the procedures can be performed rapidly. In the present work we have utilized this highly purified [³H]UCB [14] to determine the K'_f of UCB for delipidated HSA using serial ultrafiltration [15], and then utilized the same batches and concentrations of HSA and [³H]UCB and the same buffer systems to study UCB uptake by rat liver plasma membrane vesicles. The binding studies reveal effects of HSA

Abbreviations used: HSA, human serum albumin; UCB, unconjugated bilirubin; $[B_f]$, free bilirubin concentration; $[B_t]$, total bilirubin concentration; K'_f , affinity constant for bilirubin; BSP, sulphobromophthalein; OA, organic anion; LPMV, liver plasma membrane vesicles.

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concentration and the presence of KCl on the K'_r of UCB for HSA, and indicate a need to determine HSA binding and transport of UCB in the same experimental conditions. The uptake studies define the affinities and capacities of UCB for the electrogenic and electroneutral hepatocytic basolateral transport systems.

MATERIALS AND METHODS

Preparation and purification of [^3H]UCB

[^3H]UCB, specific radioactivity 53000 d.p.m./ μg , was prepared as described by Del Vecchio et al. [14]. Briefly, unlabelled UCB (Sigma, St. Louis, MO, U.S.A.), purified according to McDonagh and Assisi [16], was exposed for 3 weeks to tritium gas under high pressure (performed by New England Nuclear, Boston, MA, U.S.A.). The crude labelled UCB was purified twice by alkaline extraction of a chloroform solution, followed by recrystallization [16]. The last traces of polar impurities were then removed by $5 \times$ serial extraction of a chloroform solution of the UCB (about 0.9 mM) with 20 vol. of 0.1 M phosphate buffer, pH 7.0 [14]. After dehydrating the chloroform by filtration through chloroform-presaturated Whatman #40 paper and determination of the quantity and specific radioactivity of the UCB, the UCB/ CHCl_3 solution was distributed equally among vials. The quantity of UCB in one aliquot of the filtered CHCl_3 solution was measured by diazo assay according to Michaelsson [17], and the specific radioactivity was determined by radioassay of the same solution. The remaining vials were evaporated to dryness, sealed under argon, and then stored in the dark at -20°C until use (within 8 weeks). At intervals of 4 weeks, the dried UCB in one vial was dissolved in DMSO, and the diazo assay and radioassay were again performed, which confirmed the stability of the stored [^3H]UCB (specific radioactivity within $\pm 3\%$ of baseline). All these and subsequent procedures were carried out in dim light to minimize photodegradation of UCB [7].

Preparation of [^3H]UCB/HSA solutions

After opening a vial, the [^3H]UCB (5–10 μg) was dissolved in 30 μl of DMSO, and 3.0 ml of medium was added quickly. The medium contained 0.25 M sucrose, 20 mM Hepes, adjusted with Tris to pH 7.0, and 10 μM HSA (fatty acid free, catalogue no. A3782, lot no. 32H9300; Sigma). The UCB/HSA ratio varied from 0.04 to 0.68. In some studies, 50 mM KCl was substituted iso-osmotically for 100 mM sucrose. The same [^3H]UCB and HSA preparations and media were used for both the uptake and binding studies.

Bilirubin–albumin binding by ultrafiltration

The binding of partition-purified [^3H]UCB to delipidated HSA was determined by duplicate $5 \times$ serial ultrafiltrations of [^3H]UCB–HSA solutions at 25°C , through presoaked Centricon-10 devices (Amicon, Danvers, MA, U.S.A.), flushed with argon before use. After cycles 2–5, all the ultrafiltrate (0.9–1.0 ml) and 30 μl of retentate were sampled; residual retentate was then rediluted to the original 2.0 ml volume by adding medium without [^3H]UCB or HSA. After mixing and sampling 30 μl of rediluted retentate, the next spin cycle was performed. Control studies, to assess the binding of [^3H]UCB to the walls and frit of the Centricon device, were performed in the absence of HSA using concentrations of free [^3H]UCB comparable with those obtained in the presence of HSA.

Of each retentate, 20 μl was used for radioassay and 10 μl was used to determine content of HSA by the bicinchonic acid

method [18], corrected for the reactivity of the Hepes buffer. Radioactivity was measured after addition of 10 ml of Ecolite scintillator solution (ICN, Costa Mesa, CA, U.S.A.), using a Beckman 8100 liquid scintillation spectrometer (Irvine, CA, U.S.A.), with automatic quench corrections using external reference standards. K'_r was calculated according to the equation $K'_r = ([B_t] - [B_b]) / ([\text{HSA}] - [B_b])$ where $[B_t]$ is the total concentration of UCB and $[B_b]$ the concentration of bound UCB. $[B_t]$ was determined from the specific radioactivity (d.p.m./ml) in the filtrate, $[B_b]$ from the mean counts (d.p.m.) in the retentate before and after each spin, and $[B_b]$ as $([B_t] - [B_b])$. $[\text{HSA}]$ was calculated from the mean albumin concentration in the retentate sampled before and after each spin. In the absence of HSA, 10–20% of [^3H]UCB adsorbed to the Centricon on the first spin. The Centricon thus became saturated with [^3H]UCB, so that for subsequent spins, concentrations of [^3H]UCB in the filtrate were $97 \pm 3\%$. Correction for binding to the Centricon was thus not necessary after the first spin, and mean binding constants (K'_r) at each $[\text{HSA}]$ were calculated from the average of the stable values obtained from spins 2–5. Samples with calculated $[B_t]$ above saturation (≥ 65 nM) and occasional late samples in which the proportion of filtered label rose dramatically owing to degradation of [^3H]UCB were discarded.

Preparation of liver plasma membrane vesicles (LPMV)

Female Wistar rats (60–90 days old, 200–250 g) were allowed free access to a standard laboratory chow (Nissan, Milan, Italy) and tap water, and were housed in a constant temperature–humidity environment, with alternating 12 h cycles of light and darkness. Livers were harvested at 08:00 h for preparation of LPMV, as described previously [18]. The medium contained 0.25 M sucrose plus 20 mM Hepes adjusted with Tris to pH 7.4. The vesicles were stored in liquid nitrogen until use (within 4 weeks). The purity of LPMV was determined by measuring the activity of Na^+/K^+ -ATPase [19] and 5'-nucleotidase [20]. The release of inorganic phosphate was measured according to Widnell [21]. Protein concentration was assessed by the bicinchonic acid protein assay [22]. The relative specific activities of Na^+/K^+ -ATPase and 5'-nucleotidase were enriched 13 and 5 times respectively, indicating that the LPMV were derived mainly from the basolateral domain of the hepatocytes.

UCB transport by LPMV

Frozen LPMV were rapidly thawed at 37°C and then placed on ice for 15 min before use. For each incubation, 25 μl of LPMV suspension (150–200 μg of protein) was added to 100 μl of the buffered UCB–HSA solution. After incubation for various time intervals, either at 4 or 25°C , 1.0 ml of ice-cold stop solution, consisting of medium without KCl, UCB or HSA, was added. Each sample was rapidly filtered through nitrocellulose membranes of pore size 0.45 μm (HAWP 0.24; Millipore, Bedford, MA, U.S.A.) that had been presoaked in the stop solution for 30 min at 4°C . The filters were then washed three times with 2.5 ml of ice-cold stop solution and dried at room temperature. Electroneutral and electrogenic UCB uptake were measured as described previously for BSP [18]. To study electrogenic uptake, 50 mM KCl was substituted iso-osmotically for sucrose in the medium, and valinomycin, dissolved in methanol (50 $\mu\text{g}/\text{mg}$ of protein), was added at time 0 to both media with and without KCl. In preliminary experiments, addition of the same concentration of methanol had no effect on transport activity. Radioactivity retained on the filters was measured in a liquid scintillation spectrometer (Beta V; Kontron, Milan, Italy), after

addition of 10 ml of scintillation medium (Lipotron; Kontron). Quench corrections were automatically performed using external reference standards. Total radioactivity was corrected for [^3H]UCB retained upon filtration of the UCB-HSA solution in the absence of LPMV (0.5–1%). Valinomycin did not affect retention of UCB on the filter.

Non-specific binding of UCB to LPMV was determined in each experiment in samples containing 25 μl of LPMV and 100 μl of ice-cold incubation medium, incubated at 4 °C for the preset time; transport of UCB was determined at 25 °C. The 4 °C value was subtracted from that obtained at 25 °C, and the difference was considered as specific uptake of UCB. At a given [B_i] value, the apparent uptake values at 4 °C were stable up to 180 s. All determinations were performed at least in triplicate using two or more separate membrane preparations. The coefficient of variation was 4% for a given vesicle preparation and 9% among different vesicle preparations.

The osmolality of the medium was changed by appropriately adjusting the sucrose concentration.

Kinetic analysis of UCB uptake

UCB transport was determined mainly at [B_i] below the saturation value of 65 nM [9], in the presence of 10 μM HSA, with total UCB/HSA ratios of 0.15–0.50. The time course of UCB uptake was also assessed by serial determination of uptake at 5–30 s intervals during incubation for 300 s. Either in the presence or absence of a membrane potential, uptake of [^3H]UCB was linear up to 15 s, and, accordingly, the 15 s sampling time was chosen to determine initial uptake rates. Additional studies were performed at supersaturated concentrations of unbound UCB (80–250 nM), and with [B_i]/[HSA] ratios > 0.50, to evaluate the effects of UCB supersaturation on uptake.

Trans-stimulation of UCB uptake by BSP

LPMV (1.5–2.0 mg of protein) were preloaded with BSP (Sigma) by incubation for 30 min at room temperature in 2.0 ml of 250 mM sucrose plus 20 mM Hepes (adjusted with Tris to pH 7.0) containing 20 μM BSP. LPMV were then washed three times in 10 ml of medium without BSP and centrifuged at 70000 g for 40 min at 4 °C. UCB uptake by BSP-loaded LPMV was then measured at [B_i] ranging from 30 to 65 nM in the presence of 10 μM HSA, as described above. Control experiments were performed with LPMV subjected to the same procedure in the absence of BSP.

Statistics

All results are expressed as means $\pm$ S.D. Linear regressions of $1/K'_\text{f}$ versus [HSA] were used to determine the fit of the data to a rectangular hyperbolic function, $K'_\text{f} \times [\text{HSA}] = \text{a constant}$. K_m and V_max were determined from a computer-assisted program (SigmaPlot; Jandel Scientific, San Rafael, CA, U.S.A.) according to the Michaelis–Menten equation. The significance of differences in K_m and V_max and K'_f values was assessed with a two-tailed Student's t test for unpaired data.

RESULTS

Bilirubin–albumin binding

When K'_f was determined at a fixed [HSA] of 45–50 μM , with or without KCl, K'_f was unrelated to variations in [B_i]/[HSA] ratio from 0.08 to 0.35. By contrast, in both the presence and absence of 50 mM KCl, at [B_i] below saturation (≤ 65 nM) and [B_i]/[HSA] ratio below 0.58, K'_f (litre/mol) exhibited a hyper-

Figure 1 Effect of equilibrium total [HSA] and presence of 50 mM KCl on the association constant (K'_f) of UCB with defatted HSA

Effect was determined by serial ultrafiltration at 25 °C in a medium containing 0.25 M sucrose and 20 mM Hepes, pH 7.0. Each point represents a single cycle of ultrafiltration in one Centricon-10 device. Upper panel: rectangular hyperbolic relationship of K'_f to [HSA]. Lower panel: linear transformation of data in upper panel obtained by plotting K'_f versus $1/[\text{HSA}]$. In both panels, closed symbols represent studies performed in 0.25 M sucrose plus 20 mM Hepes adjusted with Tris to pH 7.0, and open symbols represent studies at the corresponding [HSA] and [B_i]/[HSA] ratio, in which 50 mM KCl was iso-osmotically substituted for sucrose. Circles represent studies performed at unsaturated [B_i] (below 65 nM) and [B_i]/[HSA] ratio below 0.58, where the secondary lower-affinity binding sites from UCB play little role [12]. In the lower panel, studies marked SS in the inset and shown as triangles were performed with higher, supersaturating values of [B_i] and/or [B_i]/[HSA] ratios above 0.58.

bolic, inverse relationship to [HSA] (μM) (Figure 1, upper panel) and an expected linear relationship to $1/[\text{HSA}]$ (Figure 1, lower panel), irrespective of the [B_i]/[HSA] ratio. In the absence of KCl, K'_f was $2.066 \times 10^6 + (3.258 \times 10^8/[\text{HSA}])$ ($r = 0.93$, $P < 10^{-30}$), whereas in the presence of 50 mM KCl iso-osmotically substituted for sucrose, K'_f was $2.077 \times 10^6 + (1.009 \times 10^8/[\text{HSA}])$ ($r = 0.81$, $P < 10^{-12}$). As shown in Figure 1, K'_f values significantly above these regression lines were obtained when [B_i] exceeded 65 nM (supersaturation) or [B_i]/[HSA] exceeded 0.57. K'_f values were significantly lower (P ranging from 0.0001 to 0.05) in the presence of 50 mM KCl. At [HSA] of 10 μM , at which most of the uptake studies were done, the K'_f values were $(3.47 \pm 0.73) \times 10^7$ litre/mol in the absence of KCl and $(1.22 \pm 0.13) \times 10^7$ litre/mol (65% less) when 50 mM KCl was substituted for sucrose ($P < 0.0001$). The KCl effect decreased as [HSA] increased, so that the apparent K'_f values in the presence or absence of KCl were not significantly different at [HSA] > 300 μM and were identical when extrapolated at infinite [HSA].

Figure 2 Time course of specific electrogenic (●) and electroneutral (○) bilirubin uptake by rat LPMV, pH 7.0

[³H]UCB transport was measured at specified time intervals as the difference between uptake velocities at 37 and 4 °C. Concentrations were 10 μ M HSA and 2.4 and 4.8 μ M total UCB for the electrogenic and electroneutral components respectively, yielding calculated unbound UCB concentrations of 21–22 nM in the system. Each point is the mean $\pm$ S.D. of three experiments. Inset: [³H]UCB uptake measured at 5, 10 and 15 s in electrogenic (●) and electroneutral (○) conditions. The uptake was linear. Please note that for graphical reasons the 5 s point was omitted in the time course up to 300 s.

[³H]UCB uptake by LPMV

Figure 2 shows the time course of specific [³H]UCB uptake by LPMV measured as the difference between uptake values measured at 25 and 4 °C. As shown in the inset, UCB uptake was linear for 15 s for both components (electroneutral and electrogenic). Uptake by the electroneutral component attained a plateau after 90 s. In contrast, when uptake was measured in the presence of inwardly directed K⁺ gradient upon addition of valinomycin (electrogenic component), a clear overshoot was observed at 30 s, with a rapid decline to 60 s. As with the electroneutral component, a plateau was attained after 90 s, with uptake values almost twice those of the electroneutral component. The higher values observed at equilibrium (after 90 s) in the presence of K⁺ (electrogenic component) may be accounted for by a precipitation of UCB inside the vesicles where HSA is absent. This conclusion is supported by the finding that increasing the potassium concentration outside to 100 or 200 mM led to a much higher precipitation of UCB on the filters (10 and 17% respectively) in spite of the presence of albumin. This conclusion is further supported by parallel studies we have performed by using [¹⁴C]bilirubin that indicated that addition of 50 mM KCl decreased the bilirubin solubility by 20% as assessed by its partition coefficient in chloroform (results not shown).

UCB uptake, measured at [B_i] of 50 nM, was linearly correlated with the reciprocal osmolarity (1/osM) of the outer medium ($y = 24 + 7.8x$, $r^2 = 0.93$, $P < 0.001$) indicating that UCB uptake occurred into an osmotically sensitive space. By extrapolating the value at infinite sucrose concentration (1/osM = 0), theoretically representing zero intravesicular space, it could be calculated that 40% of UCB is absorbed by the vesicles while 60% is transported into an osmotic-sensitive space.

When the specific initial uptake rate of [³H]UCB was measured at 15 s over a range of free UCB concentrations, both the electroneutral and electrogenic components exhibited saturation kinetics at [B_i] up to 70–80 nM (Figure 3). At [B_i] $\geq$ 80 nM, however, uptake rates of both the electroneutral and the electrogenic components were linearly related to free UCB concentration

Figure 3 Initial rates of electroneutral and electrogenic bilirubin uptake by rat LPMV, pH 7.0, as a function of increasing [B_i]

Concentrations were 10 μ M HSA and the UCB/HSA ratio varied from 0.15 to 0.80, yielding calculated unbound UCB concentrations from 10 to 246 nM. Each point is the mean $\pm$ S.D. of three experiments. Specific [³H]UCB transport at 25 °C (inset) was measured at 15 s as the difference between total uptake at 25 °C (■) minus non-specific binding at 4 °C (□) (pmol of UCB/mg of protein). (A) Electroneutral uptake. (B) Electrogenic component. Note that in both panels the linear portion of uptake at 25 °C at [B_i] higher than 70 nM was parallel to the line obtained at 4 °C.

($r^2 > 0.99$ for both), but the slope was significantly greater for the electrogenic uptake (0.91 versus 0.22, $P < 0.001$). The slopes of these linear components were virtually identical to those obtained from the uptake values measured at 4 °C (0.93 and 0.26 for the electrogenic and electroneutral components respectively). The higher slope observed for the electrogenic component may be related to the lower solubility of UCB in the presence of cations, particularly at supersaturating concentrations of UCB [23]. When the linear portion of the uptake process was subtracted from the initial uptake velocities, a clear saturative function was derived. The apparent K_m of the electrogenic transport was about half that obtained for the electroneutral portion (57 ± 8 versus 28 ± 7 nM respectively, $P < 0.001$); both K_m values were in the range of free UCB concentrations in plasma. The V_{max} for uptake measured in the presence of a membrane potential was almost 2.5-fold greater than that obtained for electroneutral uptake

Table 1 Effect of preloading of rat LPMV with BSP on electrogenic and electroneutral UCB uptake, at pH 7.0 and 37 °C

Vesicles were preincubated with 20 μ M BSP for 30 min, and BSP was then removed by washing (see the Materials and methods section). UCB uptake was measured in the presence of 10 μ M HSA, at a total UCB/HSA ratio of 0.50, yielding a calculated unbound UCB concentration of 30 nM for electroneutral and 65 nM for electrogenic uptake (due to the presence of KCl). Data are expressed as means $\pm$ S.D. of three different experiments.

LPMV preparation	$[^3\text{H}]\text{UCB}$ uptake (pmol $\cdot$ mg $^{-1}$ of protein $\cdot$ 15 s $^{-1}$)	
	Electroneutral uptake	Electrogenic uptake
Control	41.0 $\pm$ 3.2	52.7 $\pm$ 5.9
Preloaded with BSP	70.8 $\pm$ 1.0	85.4 $\pm$ 7.8
<i>Trans</i> -stimulation (%) ...	+72 $\pm$ 6*	+61 $\pm$ 7*

* $P < 0.001$

(112 $\pm$ 12 versus 45 $\pm$ 4 pmol $\cdot$ mg $^{-1}$ of protein $\cdot$ 15 s $^{-1}$ respectively, $P < 0.001$).

To investigate if UCB was transported by the same mechanisms responsible for the hepatocytic uptake of BSP, *trans*-stimulation experiments were performed. This experimental approach was selected since the demonstration of a direct competition by BSP on the uptake of UCB was unfeasible, as the addition of BSP outside the vesicles would alter the value of the UCB K'_t due to competition of the dye with albumin. As shown in Table 1, both the electroneutral and electrogenic components of UCB uptake were *trans*-stimulated by about two-thirds when vesicles were preloaded with BSP.

DISCUSSION

Our data show for the first time that UCB is transported across the basolateral plasma membrane vesicles of rat hepatocytes by at least two systems, one sensitive and one insensitive to the membrane potential. The uptake rate was linearly correlated with the intravesicular volume, indicating that uptake rather than binding was measured. Both systems follow saturation kinetics with respect to $[\text{B}_i]$ below saturation (≤ 65 nM) [9]. At $[\text{B}_i]$ above saturation, the uptake rates are linearly related to free UCB concentrations, suggesting possible diffusion and/or precipitation of UCB complexes. Both the affinity (apparent K_m) and the uptake capacity (V_{max}) for unbound UCB differed between the two uptake systems; the electroneutral system showed a higher affinity but a lower capacity for UCB than the electrogenic system. Interestingly, the apparent K_m of each system was in the range of free UCB concentrations in plasma [13] and below the saturation concentration for unbound UCB (75 nM) at pH 7.4 [9].

This study emphasizes that, in order to determine the relationship between transport and unbound UCB concentrations $[\text{B}_i]$, it is important to independently determine the binding affinity (K'_t) of the same batches of UCB and HSA, at the same concentrations and in the same media used for the uptake studies. Only the binding constants thus derived are directly applicable to calculate the $[\text{B}_i]$ in the uptake system. It is clearly inappropriate to use the accepted value of the association constant (K'_t) of UCB to HSA [12,13] obtained by a different method, under different conditions of pH and ionic strength, using different buffers without sucrose in the medium. This accepted K'_t value was over an order of magnitude higher than that determined in our experimental system.

Another factor in the discordant K'_t values may be that the

peroxidase studies were performed with systems supersaturated with unbound UCB [9]. The insoluble, microparticulate phase of excess unbound UCB may have not reacted with the peroxidase and thus been falsely excluded from the unbound fraction, resulting in an overestimation of K'_t . Our ultrafiltration studies likewise yielded high K'_t values when $[\text{B}_i]$ exceeded saturation, probably because the insoluble excess of unbound $[^3\text{H}]\text{UCB}$ did not filter and was thus excluded from the apparent $[\text{B}_i]$ determined by radioassay in the ultrafiltrate.

Our lower K'_t values might have been due in part to an artefactual increase in the apparent $[\text{B}_i]$, related to tritium exchange from $[^3\text{H}]\text{UCB}$ to ultrafiltrable water [24], or to the formation of polar oxidation products of $[^3\text{H}]\text{UCB}$ that are less well bound to HSA [25]. In order to minimize ^3H exchange and oxidation of UCB, we performed our studies at pH 7.0 [25], in dim light under argon [7], and completed the five cycles of ultrafiltration in less than 120 min. The stability of the K'_t values over the last three or four serial cycles in each study suggests that major ^3H exchange or oxidation had not occurred, and that the filtration device had become saturated with unbound UCB.

The uptake studies lasted less than 6 min, and determining initial uptake velocity lasted only 15 s. The $< 0.01\%$ of labelled polar UCB derivatives would cause little error in assessment of uptake, even though binding of such derivatives to albumin is much lower [25]. The uptake values, moreover, were corrected for the precipitation of UCB on the filters in the absence of vesicles. To yield the specific uptake rates that were used for calculating the kinetic constants, the uptake rates were further corrected for 'non-specific' binding of UCB to LPMV as determined at 4 °C.

We found that the binding affinity of UCB for albumin, determined in our ultrafiltration studies, was profoundly affected by both albumin concentration and the presence of KCl. K'_t was inversely related to $[\text{HSA}]$, resulting in a greater proportion of unbound UCB as $[\text{HSA}]$ increased. Similar effects have been reported with other OAs and hormones [26–29]. This may in part explain the well-known enhanced hepatic uptake of OAs that occurs with increasing $[\text{HSA}]$ [10,11], which had been postulated to result from conformational changes in HSA at the hepatocyte surface or the effects of the unstirred water layer [10]. We propose that this decrease in affinity for UCB results from the known progressive self-aggregation of HSA [30] to form oligomers in which the binding sites for OAs are altered in conformation and/or accessibility.

The effect of KCl in decreasing the binding of UCB to HSA has been reported previously [31–33], and is probably due to competitive inhibition by Cl^- of the binding of the UCB dianion to the cationic arginine residues at the binding sites in the HSA ligand pockets [30]. This could account for the finding that added Cl^- appears to stimulate electroneutral uptake of organic anions only if albumin is present [34]. It might also account, at least in part, for the enhanced hepatocytic uptake in the presence of an inwardly directed positive transmembrane potential gradient produced by adding KCl (electrogenic uptake) [35]. In our studies, we took into account the increase in $[\text{B}_i]$ in the presence of KCl, and thus clearly confirmed the presence of an electrogenic component. The overshoot phenomenon observed in the presence of an inwardly directed positive gradient further confirmed that part of the UCB transport is sensitive to membrane potential, as observed for other OAs [5,6].

The intravesicular pH was pH 7.4, and the external medium was at pH 7.0, to minimize ^3H exchange and degradation of $[^3\text{H}]\text{UCB}$. The degrees of ionization of the unbound UCB species at the pH values of each phase [8,9] indicate, however, that this pH gradient is unlikely to play a significant role in the uptake of

UCB. Thus, if UCB diacid (B^{2-}) is the transported species, this species accounts for 95 % of unbound UCB at pH 7.0 and 85 % at pH 7.4, a 10 % difference. If UCB monoanion (HB^-) is the transported species, the proportion of this species is 8 % of unbound UCB at pH 7.0 and 15 % at pH 7.4, a 2-fold difference with the gradient opposing uptake.

In the intact rat, UCB has been shown to share uptake mechanisms with both BSP and Indocyanine Green [36]. Our *trans*-stimulation studies with BSP indicated that both the electroneutral and electrogenic uptake mechanisms are shared by UCB and BSP. The kinetic characteristics of UCB uptake, however, differed from those of BSP, for which the capacities of the two systems were similar but the affinities differed [19]. Though the studies with BSP were performed in the absence of albumin, since BSP is sufficiently water soluble, recent evidence suggests that hepatocytic uptake of BSP, like UCB, is driven by the free concentration of the ligand [11], thus rendering valid comparison of the two studies.

In summary, these first direct studies of UCB transport at $[B]$ below saturation indicate that UCB uptake is operated mainly by two distinct mechanisms, one insensitive and the other sensitive to membrane potential. The former shows high affinity and low capacity whereas the latter has higher capacity but lower affinity for the natural ligand. The relative contributions of any of the four putative carrier proteins for OAs [1–4] remain to be investigated. Further studies are needed with $[^{14}C]UCB$ to ensure that the K_t values and uptake rates obtained with $[^3H]UCB$ are not confounded by tritium exchange. Studies are also needed to determine whether the effects of KCl are due to an increase in ionic strength or to K^+ or Cl^- ions, and if the binding of other OAs (e.g. BSP) to HSA is similarly affected by KCl. If so, re-evaluation will be required of published K_m and V_{max} values for the relationships between hepatic uptake rates and unbound concentrations of these OAs. In any case, all such future studies must determine the binding of OAs to HSA and the uptake rates using the same media concentrations and batches of the components, and must work at concentrations of the unbound ligand below saturation.

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