Kinetics of hepatic uptake of unconjugated bilirubin

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Summary
1. The uptake of bilirubin was studied in the perfused rat liver by a multiple-indicator dilution technique employing the three-compartment model of Goresky.
2. The kinetics of hepatic bilirubin uptake could be described by the Michaelis-Menten equation.
3. The maximal uptake velocity ($V_{max}$) and the apparent half-saturation constant ($K_m$) were $4.4 \pm 0.5$ nmol s$^{-1}$ g$^{-1}$ of liver and $58 \pm 16$ nmol/g of liver respectively, indicating that the hepatic uptake system for bilirubin under normal conditions is operating far below saturation.
4. Sodium taurocholate did not compete with bilirubin for hepatic uptake.
5. These findings are consistent with the concept that carrier-mediated transport is responsible for hepatocellular uptake of bilirubin and that bilirubin and bile acids enter the hepatocyte via separate pathways.

Key words: bile acids, bilirubin, liver.

Introduction
The mechanism by which bilirubin enters the hepatocyte cannot be fully explained by the relative binding affinities of bilirubin for albumin, its transport protein in the blood, and ligandin, its binding protein in the hepatocytic cytosol (Bloomer, Berk, Vergalla & Berlin, 1973a). It has therefore been suggested that the uptake process requires the participation of the plasma membrane (Bloomer et al., 1973a). Kinetics of hepatic uptake for other organic anions such as bromosulphthalein (Goresky, 1964), Indocyanine Green (Paumgartner, Probst, Kraines & Leevy, 1970; Paumgartner & Reichen, 1975) and taurocholate (Reichen & Paumgartner, 1975; Glasinovic, Duval & Erlinger, 1975) have been found consistent with the hypothesis that these substances are taken up into the hepatocyte by carrier-mediated transport. The kinetics of bilirubin uptake, however, have not adequately been defined. Furthermore, it is unclear whether bilirubin and bile acids, the major organic anions in bile, enter the hepatocyte via a common or by separate pathways. Hepatic bilirubin uptake was therefore studied in the perfused rat liver. In particular, the problem whether bilirubin and taurocholate compete for uptake by the hepatocyte was investigated.

Materials and methods

Animals and chemicals
Male SPF-rats of the Sprague-Dawley strain, weighing 372–472 g, maintained on a standard rat diet (Altromin 300 R, Altromin GmbH, Lage, Germany) and tap water ad libitum, were used as liver donors. Chromatographically pure bovine serum albumin (Behringwerke AG, Marburg/Lahn, Germany), analytical grade crystalline bilirubin (Serva Entwicklungslabor, Heidelberg, Germany), analytical grade sodium taurocholate (Calbiochem, Lucerne, Switzerland) and sodium carboxy-14C-labelled taurocholate (International Chemical and Nuclear Corp., Calif., U.S.A.) were used as provided
by the manufacturers. $^{99m}$Tc-labelled albumin was prepared by Dr H. Roesler from the Division of Nuclear Medicine (Inselspital, Berne, Switzerland). Less than 2% of the pertechnetate was in its free form, as determined by equilibrium dialysis.

**Perfusion experiments**

The operation and perfusion techniques have been described previously (Herz, Cueni, Bircher & Paumgartner, 1973). The perfusion medium consisted of Krebs-Ringer bicarbonate buffer, which contained 20% (v/v) bovine erythrocytes washed five times with sodium chloride solution (150 mmol/l), bovine serum albumin (294 μmol/l) and glucose (5-55 mmol/l). The livers were perfused with a mean perfusion pressure of 10.8 ± 0.4 cm water and a mean rate of 1.47 ± 0.09 ml min⁻¹ g⁻¹ of liver in a constant-temperature cabinet at 37 ± 0.2°C. The pH, standard bicarbonate, $P_{CO_2}$ and base excess of the perfusate were monitored during the perfusion experiment and acid-base imbalance was corrected with sodium bicarbonate. The viability of the perfused rat liver preparation was tested by measurements of bile production throughout the experiment and by measurements of the potassium concentration in the perfusate at the beginning and at the end of the experiment. Bile production was 0.98 ± 0.13 and 0.88 ± 0.11 μl min⁻¹ g⁻¹ at the beginning and at the end of the perfusion experiments respectively. The corresponding values for the potassium concentration in the perfusate were 5.3 ± 0.5 and 5.7 ± 0.3 mmol/l. Furthermore, the structural integrity of the liver was examined by light and electron microscopy. Apart from a slight increase of autophagic vacuoles, no pathological changes were found. All variables studied were related to the liver weight determined at the end of each perfusion experiment. The liver weight averaged 3.2 ± 0.3% of the body weight.

All indicators were rapidly injected, in a constant volume of 210 μl, into the portal vein. Immediately after the injections, the total hepatic venous outflow was collected in 2 s periods up to 40 s in tared tubes and weighed. Thereafter the outflow was further collected up to 2 min, to eliminate recirculation of the indicators.

At the beginning and at the end of each of eight perfusion experiments, a mixture of $^{51}$Cr-labelled erythrocytes (1.35 μCi) and $^{99m}$Tc-labelled albumin (15 μCi) was injected into the portal vein to determine the intra- and extra-vascular space of the liver. Between these two injections, four doses (50–2500 nmol) of bilirubin, alone ($n = 15$) or mixed with an equimolar amount of sodium [14C]taurocholate ($n = 9$), were injected together with 15 μCi of $^{99m}$Tc-labelled albumin (extravascular reference indicator) at 20 min intervals. The different doses were applied in a random order although no difference was apparent whether bilirubin was given at the beginning or at the end of a perfusion experiment. The bilirubin solutions were prepared immediately before use by dissolving bilirubin in sodium carbonate (100 mmol/l) and diluting this stock solution with appropriate volumes of erythrocyte-free perfusate containing albumin at a concentration of 294 μmol/l.

**Analytical procedures**

$^{99m}$Tc and $^{51}$Cr radioactivity were counted in a Packard 3002 Autogamma spectrometer. $^{99m}$Tc radioactivity was determined in the supernatant immediately after the perfusion experiment. $^{51}$Cr radioactivity was determined in whole, haemolysed blood after decay of the $^{99m}$Tc radioactivity for at least ten half-life times. 14C radioactivity was counted in the supernatant in a Packard Tricarb 3380 liquid-scintillation counter with Instagel (Packard Instrument Co. Inc., Ill., U.S.A.) as scintillator. The counting efficiency was determined by the channel ratio method employing an external standard. Bilirubin was measured by a micromodification of the Malloy-Evelyn procedure (Abrams & Elliot, 1974). The coefficient of variation of this method, in the range of concentrations measured (1.5–150 μmol/l), was 4.6%.

**Mathematical and statistical analysis**

The distribution spaces of the different indicators were calculated from the mean transit times and the flow rates according to Meier & Zierler (1954). The sinusoidal volumes were calculated according to Goresky (1963) after subtraction of the volume of the large, non-exchanging vessels.

The uptake of bilirubin and of taurocholate was calculated with the three-compartment model proposed by Goresky, Bach & Nadeau (1973). This model includes a vascular compartment (A), an extravascular compartment (B) and a cellular compartment (C). The outflow profiles of bilirubin and
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taurocholate, substances which are taken up into C, consist of two terms, namely a throughput and a returning component. Accordingly, their outflow fraction $Q(t)$ can be expressed as in eqn. (1).

$$Q(t) = e^{-k_1 t} C + \text{returning material} \quad (1)$$

In this equation, $Q(t)_k$ is the outflow fraction of albumin, the reference indicator, which distributes in A and B but is not taken up into C; $k_1$ (dimension $t^{-1}$) is the removal rate constant describing the transport of the extracted substance into the cellular compartment, $\theta$ is the ratio C/A and $\gamma$ the ratio B/A. Thus $\theta/(1 + \gamma)$ represents the ratio of the cellular to the extracellular space. The term for the returning material in eqn. (1) was neglected since such a component could not be measured with the methodology used. If present, it must have been small compared with the throughput component. Eqn. (1) was therefore transformed into eqn. (2).

$$\ln \frac{Q(t)_k}{Q(t)_e} = \frac{k_1 \theta}{1 + \gamma} (t) \quad (2)$$

$k_1 \theta/(1 + \gamma)$ can be estimated as the slope of a plot of the natural logarithm of the ratio albumin outflow fraction/bilirubin outflow fraction versus time. Since the intravascular and the extravascular space are determined experimentally, $\gamma$ and consequently $k_1 \theta$ can be calculated.

The initial uptake velocity of bilirubin and bile acids was calculated as the product of $k_1 \theta$ and the dose. It has the dimension of nmol of bilirubin taken up s$^{-1}$ g$^{-1}$ of liver (nmol s$^{-1}$ g$^{-1}$). The relationship between initial uptake velocity ($v$) and the dose ($D$) was analysed by the Michaelis–Menten equation (Michaelis & Menten, 1913):

$$v = \frac{V_{\text{max}}D}{K_m + D} \quad (3)$$


$$\frac{D}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \quad (4)$$

The apparent half-saturation constant ($K_m$) and the maximal uptake velocity ($V_{\text{max}}$) were computed non-linearly in a weighted form according to Wilkinson (1961). For statistical comparison of different sets of kinetic data the linear transformation of the Michaelis–Menten equation given above (Dixon & Webb, 1970) was used.

Regression analysis was performed by the method of least squares. Mean values of two samples as well as regression lines were compared by Student's $t$-test after testing the equality of variances by the $F$-test. The goodness of the fit was expressed as the $F$ value, representing the ratio (regression mean square)/(residual mean square). $P<0.05$ was regarded as significant. Unless otherwise stated, all results are expressed as the mean value ± SD.

Results

As described earlier for taurocholate (Reichen & Paumgartner, 1975), unconjugated bilirubin distributed in a space (0.200 ± 0.033 ml/g) not significantly different from that of albumin (0.197 ± 0.038 ml/g). Thus albumin appeared to be an appropriate reference substance for the study of hepatic bilirubin uptake. The natural logarithm of the ratio (albumin outflow fraction)/(bilirubin outflow fraction) increased with time in a linear fashion (Fig. 1), indicating that a further requirement of the model was fulfilled, namely that during the period of observation bilirubin returning from the hepatocyte could be neglected.

When the dose of bilirubin was increased from 50 to 2500 nmol (fifteen observations in eight perfusion experiments), the bilirubin uptake velocity increased from 0.3 to 3.2 nmol s$^{-1}$ g$^{-1}$ in a non-linear fashion approaching a maximal value asymptotically (Table 1). This function could adequately be described by a rectangular hyperbola obeying the Michaelis–Menten equation (Fig. 2). Accordingly, an apparent half-saturation constant ($K_m$) of 58 ± 16 nmol/g of liver and a maximal uptake velocity ($V_{\text{max}}$) of 4.4 ± 0.5 nmol s$^{-1}$ g$^{-1}$ of liver could be calculated by the procedure of Wilkinson.

The kinetics of hepatic uptake of bilirubin were not significantly influenced by the simultaneous injection of equimolar amounts of sodium taurocholate (Table 2). Under these conditions, a $K_m$ of 80 ± 22 nmol/g and a $V_{\text{max}}$ of 4.5 ± 0.6 nmol s$^{-1}$ g$^{-1}$ were obtained for bilirubin uptake (nine observations in five perfusion experiments). When a linear transformation of the Michaelis–Menten equation (Dixon & Webb, 1970) was employed for a statistical comparison of the two sets of kinetic data, the regression line representing bilirubin uptake in the presence of taurocholate was not significantly different from that obtained in the absence of taurocholate (Fig. 3). Simultaneous measurements of taurocholate uptake in the presence of bilirubin
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**Fig. 1.** Dilution curves of $^{99}$Tc$^{m}$-labelled albumin ($\bigcirc$) and bilirubin ($\bullet$) in hepatic vein blood after a single rapid injection of 250 nmol of bilirubin into the portal vein. A plot of the natural logarithm of the ratio albumin outflow fraction/bilirubin outflow fraction ($\Delta$) versus time results in a straight line.

**Fig. 2.** Relationship between dose and uptake velocity of bilirubin. The rectangular hyperbola (\(k = 4.4D/(58 + D)\); \(F = 15.60\); \(n = 15\)) was computed according to Wilkinson (1961).

(Fig. 4) revealed Michaelis–Menten kinetics (\(K_m: 255\) nmol/g; \(V_{max}: 19\) nmol s$^{-1}$ g$^{-1}$; \(n = 9\)), not significantly different from those obtained in the absence of bilirubin (\(K_m: 235\) nmol/g; \(V_{max}: 19\) nmol s$^{-1}$ g$^{-1}$; \(n = 18\)); these data are from Reichen & Paumgartner (1975), newly calculated with the three-compartment model.

**Table 1.** Uptake of unconjugated bilirubin by the perfused rat liver

Results are mean values of seven perfusion experiments.

\(\gamma = \text{ratio extravascular/vascular compartment}; k_1 = \text{ratio cellular/vascular compartment}; k_1\theta = \text{removal rate constant.}\)

\[
\begin{array}{cccccc}
\text{Dose (nmol)} & \text{Liver wt. (g)} & \gamma & \frac{k_1\theta}{1 + \gamma} & \text{Uptake velocity (nmol s$^{-1}$ g$^{-1}$)} \\
50 & 13.7 & 0.730 & 0.132 & 0.28 \\
125 & 13.2 & 0.569 & 0.126 & 0.76 \\
125 & 12.0 & 0.942 & 0.065 & 0.72 \\
250 & 13.0 & 1.007 & 0.121 & 1.16 \\
250 & 13.7 & 0.690 & 0.070 & 0.76 \\
250 & 13.8 & 0.689 & 0.112 & 1.21 \\
500 & 13.0 & 1.737 & 0.088 & 1.43 \\
500 & 14.2 & 1.163 & 0.057 & 1.72 \\
750 & 13.2 & 0.637 & 0.051 & 1.78 \\
750 & 13.7 & 0.753 & 0.059 & 1.84 \\
750 & 12.0 & 0.830 & 0.027 & 2.02 \\
1500 & 13.7 & 0.760 & 0.064 & 3.99 \\
1500 & 12.3 & 0.603 & 0.014 & 2.98 \\
2500 & 13.0 & 0.779 & 0.030 & 3.22 \\
2500 & 13.8 & 0.728 & 0.034 & 3.05 \\
\end{array}
\]

**Discussion**

The present study indicates that the kinetics of hepatic uptake of unconjugated bilirubin do not differ qualitatively from those of other organic anions which are removed from the blood by the liver. Similar to the kinetics of bromosulphthalein (Goresky, 1964), Indocyanine Green (Paumgartner et al., 1970; Paumgartner & Reichen, 1975) and taurocholate uptake (Reichen & Paumgartner, 1975; Glasinovic et al., 1975), they are dependent on a saturable process obeying Michaelis–Menten kinetics. It appears unlikely that the smaller fractional clearance of higher doses of bilirubin has been due to toxic effects of the high concentrations of bilirubin.
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TABLE 2. Uptake of unconjugated bilirubin by the perfused rat liver when equimolar amounts of sodium taurocholate were injected together with the bilirubin
Results are mean values of four perfusion experiments. \( \gamma \), \( \theta \) and \( k_1 \) are identified in Table 1.

<table>
<thead>
<tr>
<th>Dose (nmol)</th>
<th>Liver wt. (g)</th>
<th>( \gamma )</th>
<th>( k_1 \theta )</th>
<th>Uptake velocity (nmol s(^{-1}) g(^{-1}))</th>
</tr>
</thead>
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<tr>
<td>125</td>
<td>13.0</td>
<td>0.864</td>
<td>0.154</td>
<td>0.80</td>
</tr>
<tr>
<td>250</td>
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<td>0.097</td>
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<tr>
<td>500</td>
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<td>1.10</td>
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<tr>
<td>750</td>
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<td>1.78</td>
</tr>
<tr>
<td>1000</td>
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<tr>
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<td>0.036</td>
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<tr>
<td>1500</td>
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<tr>
<td>2500</td>
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<tr>
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<td>0.738</td>
<td>0.026</td>
<td>2.76</td>
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since even the highest doses of bilirubin did not affect the factors of viability tested. Moreover, a high dose of bilirubin did not influence the subsequent uptake of a small dose. The findings are in accordance with a recent report of Scharschmidt, Waggoner & Berk (1975), who have used a clearance technique to study bilirubin uptake in the intact rat. They support the concept that carrier-mediated transport is responsible for hepatic uptake of bilirubin. Although the role of the Y and Z proteins (Levi, Gatmaitan & Arias, 1969) in this first event in the removal of bilirubin from the blood remains to be clarified, evidence is available which suggests that the uptake mechanism requires the participation of the plasma membrane (Bloomer et al., 1973a). This concept is supported by the following arguments. No transfer of \([^{14}C]\)bilirubin from albumin to the organic anion-binding protein of rat cytosol could be demonstrated in vitro by Bloomer et al. (1973a). Instead, albumin was shown to strip \([^{14}C]\)bilirubin from these proteins. This behaviour can be explained by the observation that the primary binding affinity of Y protein for bilirubin is less than that of albumin (Arias, 1972; Arias & Jansen, 1975). It must, however, be noted that Meuwissen (1975) has recently presented evidence that the affinity of bilirubin to ligandin is greater than that to albumin, when tested under ionic and pH conditions closer to those of hepatic cytosol.

The maximal initial uptake rate exceeded the reported steady-state excretory transport maximum (Weinbren & Billing, 1956; Robinson, Yannoni & Nagasawa, 1971) by a factor of about 8. In this respect, bilirubin behaves similarly to other organic anions. Thus uptake has not been found to be the rate-limiting step for the overall excretion of bromosulphthalein (Goresky, 1964), Indocyanine Green (Paumgartner & Reichen, 1975) or taurocholate.

![Fig. 3. Kinetics of bilirubin uptake in the absence (●) and in the presence (⊿) of equimolar doses of sodium taurocholate, plotted according to a linear transformation of the Michaelis–Menten equation.](image-url)
FIG. 4. Kinetics of taurocholate uptake in the absence (■) and in the presence (Δ - - Δ) of equimolar doses of bilirubin, plotted according to a linear transformation of the Michaelis-Menten equation.

(Reichen & Paumgartner, 1975) from the blood into the bile. The plasma concentration of bilirubin leading to half-saturation of the uptake mechanism was 280 µmol/l. This indicates that the hepatic uptake system for bilirubin is operating far below saturation at physiological concentrations of bilirubin in serum. Several limitations must be pointed out when the kinetic parameters for initial bilirubin uptake obtained in the perfused liver are compared with excretory transport rates measured under steady-state conditions in vivo. Obviously, the conditions of the isolated liver perfusion may reduce hepatocellular function (Paumgartner, Herz, Sauter & Schwarz, 1974). More importantly, however, the composition of the perfusion medium may influence hepatic uptake of bilirubin.

The difference between the albumin concentration in rat plasma and in the perfusate used in the present studies warrants special consideration. Bilirubin is tightly bound to serum albumin (Jacobson, 1969) and its clearance by the perfused rat liver is related to its non-albumin-bound fraction rather than to its total concentration in the perfusate (Barnhart & Clarenburg, 1973a). Consequently, its clearance by the perfused rat liver is enhanced when the albumin concentration in the perfusate is lowered (Barnhart & Clarenburg, 1973a). Although the normal albumin concentration in rat serum is about 441 µmol/l (Schultz, Jamison, Shay & Greenstein, 1954), the perfusate used in the present studies contained only 294 µmol of albumin/l. This difference in albumin concentration may partly explain why the \( V_{max} \) observed in the present study exceeded that reported by Scharschmidt et al. (1975), who studied bilirubin uptake in the intact rat by a clearance technique. Another factor contributing to this difference may be that with the rapid-injection multiple indicator dilution technique in the isolated perfused liver it is possible to measure bilirubin uptake at a time when the hepatocyte is virtually empty of bilirubin, which is not possible with a technique measuring plasma disappearance of bilirubin in the intact rat.

Finally, it must be considered that the measurements of bilirubin uptake may be influenced by the binding of bilirubin to erythrocytes. This phenomenon has been described in vitro by Barnhart & Clarenburg (1973b), who demonstrated that washed bovine erythrocytes (13%, v/v) in Krebs-Ringer bicarbonate buffer (pH 7.4), containing 220 µmol of bovine albumin/l, rapidly and reversibly bind 7.3% of the bilirubin present. In accordance with these findings, about 9% of the bilirubin was bound to erythrocytes under the conditions of our experiments, in which a perfusate containing 20% (v/v) bovine erythrocytes and 294 µmol of bovine albumin/l was used. Since this extent of binding to erythrocytes only leads to a minor erythrocyte carriage phenomenon (Goresky, 1975; Goresky, Bach & Nadeau, 1975), and since the mathematical problems associated with an analysis of this effect have not yet been
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resolved, no correction for erythrocyte carriage of bilirubin was attempted.

For the biliary excretion of organic anions multiple mechanisms have been suggested (Alpert, Mosher, Shanske & Arias, 1969). Similarly, the uptake of anionic dyes and bile acids by the hepatocyte may also involve separate pathways. Thus taurocholate does not inhibit the uptake of Indocyanine Green by the perfused rat liver (Paumgartner & Reichen, 1975) and glycocholate has no influence on the initial clearance of Indocyanine Green (Paumgartner et al., 1970) and bilirubin (Scharschmidt et al., 1975) in the intact rat. Dehydrocholate, a synthetic trioxocholanic bile acid, inhibits the transport of bilirubin and Indocyanine Green from plasma to bile in man but has no effect on the initial plasma disappearance rate of these anionic dyes (Bloomer, Boyer & Klatskin, 1973b). The lack of competition between taurocholate and unconjugated bilirubin for uptake by the hepatocyte observed in the present study supports the concept that both anions enter the hepatocyte separately rather than by a common pathway. These findings could also explain the observation that under certain conditions, such as Gilbert's syndrome, the uptake of unconjugated bilirubin is impaired (Berk, Bloomer, Howe & Berlin, 1970) whereas bile acid transport appears to be unaffected (Schwarz, Paumgartner & Preisig, 1975).

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