Hepatocytes internalize high density lipoprotein (HDL) at the basolateral membrane. Most HDL is recycled while some is shuttled to the canalicular membrane by transcytosis. Here, transport of HDL was analyzed by mathematical modeling based on measurements in polarized hepatic HepG2 cells. Recycling of HDL from basolateral sorting endosomes was modeled by applying the rapid equilibrium approach. Analytical expressions were derived, which describe in one model the transport of HDL to the subapical compartment/apical recycling compartment, the biliary canaliculus (BC), and to late endosomes and lysosomes (LE/LYS). Apical endocytosis of HDL predicted by the model was confirmed for rhodamine-dextran and fluorescent asialoorosomucoid, markers for LE/LYS in living HepG2 cells. Budding of endocytic vesicles from the BC was directly observed by time lapse imaging of a fluorescent lipid probe. Based on fitted kinetic parameters and their covariance matrix a Monte Carlo simulation of HDL transport in hepatocytes was performed. The model was used to quantitatively assess release of HDL-associated free cholesterol by scavenger receptor BI. It is shown that only 6% of HDL-associated sterol reaches the BC as a constituent of the HDL particles, whereas the remaining sterol is rapidly released from HDL and shuttled to the BC by non-vesicular transport.

Hepatic transport of high density lipoprotein (HDL) and processing of its associated lipids including sterols is a key step during reverse cholesterol transport. Free cholesterol and cholesteryl esters (CE) are transferred from the HDL particle to the hepatocyte (1–3). Biliary secretion of cholesterol along with bile salts and phosphatidylcholine (PC) is the only pathway of sterol clearance from the body (4). Uptake of sterols from HDL in the hepatocyte is partially independent of the processing of the HDL protein moiety. This so-called selective uptake is mediated largely by the scavenger receptor BI (SR-BI) at the basolateral membrane of hepatic cells (3, 5). It has been proposed that endocytosis and recycling of the whole HDL particle occurs in hepatocytes and contributes to selective CE uptake (6–8). Recently, we demonstrated that the natural cholesterol analog dehydroergosterol (DHE) incorporated into fluorescent HDL is rapidly released from the lipoprotein at the basolateral membrane of polarized HepG2 cells (9). This process required SR-BI but was not dependent on metabolic energy in contrast to uptake of the HDL particles. DHE was rapidly shuttled to the apical canalicular membrane of HepG2 cells by ATP-independent non-vesicular transport (9, 10). The canalicular membrane of HepG2 cells forms apical closed vacuoles that resemble the biliary canaliculi (BC) of hepatocytes in many respects (11). We found evidence for internalization of DHE and fluorescent PC from the BC of HepG2 cells (10, 12). We demonstrated that fluorescent PC and DHE become delivered to a subapical compartment or apical recycling compartment (SAC/ARC) in vesicles in 15–30 min. The identity of this SAC/ARC was shown by colocalization with fluorescent transferrin (TF) as well as fluorescent sphingomyelin, a marker for lipid transcytosis in polarized HepG2 cells (10, 13).

By labeling HDL with the fluorescent dye Alexa-488 (Alexa-488-HDL) and employing quantitative fluorescence microscopy, we demonstrated rapid internalization and recycling of fluorescent HDL at the basolateral membrane of polarized HepG2 cells. Alexa-488-HDL became internalized into TF-containing vesicles and accumulated in the SAC/ARC within 10 min. About 50–80% of Alexa-488-HDL rapidly recycled and became released from the cells. About 3% of initial Alexa-488-HDL were exported to the BC within a 30-min chase, whereas about 2% were found in late endosomes and lysosomes (LE/LYS) after prolonged incubation (9). By quantitative fluorescence imaging we were able to measure detailed time courses of HDL transport in HepG2 cells. From studies in hepatocyte couplets it has been suggested that HDL is bound to SR-BI and the receptor-ligand complex accumulate in a SAC/ARC as we reported for HDL in polarized HepG2 cells (5, 9). Thus, trafficking of HDL is intimately linked to transport of SR-BI. It can be expected that mechanisms underlying SR-BI expression and transport regulate also HDL transport in hepatic cells.

Detailed understanding of the pathways and dynamics of transport of HDL in hepatic cells is of crucial importance to analyze the processing of HDL apoprotein and sterol in the intact organ. However, even if itineraries and kinetics of intra-
cellular HDL transport are known, the connection between pathways and rate-limiting transport steps remain obscure. Mathematical modeling is a powerful tool to analyze intracellular transport, once transport kinetics to cellular compartments have been determined. Here, a detailed mathematical analysis of intrahepatic HDL transport based on our previous measurements in polarized HepG2 cells has been performed (9). A model was developed which predicts that most HDL is recycled from basolateral sorting endosomes (SE) while a portion is exported to the BC via the SAC/ARC. Analytical solutions for a 4-compartment model were derived and fitted simultaneously to the experimental data for transport of fluorescent HDL to the BC, to the SAC/ARC as well as to LE/LYS. Apical endocytosis of HDL and transport to LE/LYS was included in the model based on experimental results on targeting of lysosomal markers from the BC to LE/LYS in HepG2 cells. In addition endocytosis from the BC was directly visualized by time-lapse imaging of a fluorescent lipid probe in living HepG2 cells. Rate constants for all HDL transport steps are provided and used in a Monte Carlo simulation. The model for HDL transport was applied to determine the extent of SR-BI-mediated sterol release from the HDL particles based on quantitative trafficking data for HDL-associated DHE in HepG2 cells.

**EXPERIMENTAL PROCEDURES**

Reagents—NBD-labeled PC 1-palmitoyl-2-[6-(7-nitro-2-1,3-benzoxadiazol-4-yl)aminocaproyl]-sn-glycero-3-phosphatidylcholine (C6-NBD-PC) was obtained from Avanti Polar Lipids (Birmingham, AL). 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC12), rhodamine-labeled dextran (70 kDa) (Rh-dextran) and Alexa-488-Tf for 1, 15, or 30 min at 37 °C. Cells were washed and imaged as fluorescence of Rh-dextran, TexRed-ASOR, and DiIC12 in the BC was quantified by defining regions of interest (ROI) for this compartment. In the case of Rh-dextran and DiIC12 integrated fluorescence intensity in ROI was measured after background subtraction and normalized to total cell-associated fluorescence in the two cells forming a BC. The ratio \( R(t) \) of the integrated fluorescence intensity of ROI \( U_{ROI}(t) \) and of whole cells \( U_{cell}(t) \) was then calculated and plotted as function of time as described (9, 10, 12). For TexRed-ASOR mean fluorescence in the BC was determined and plotted as function of total chase time (labeling + chase).

**Data Analysis**—BC-associated fluorescence of Rh-dextran, TexRed-ASOR, and DiIC12 was evaluated and plotted using SigmaPlot 4.0 (SPSS Inc., Chicago, IL). The equations for the HDL transport model were used to derive rate constants for transport of HDL between various compartments. Curve-fitting of these equations was done by a multicompartiment nonlinear regression procedure implemented in the SAAM software (SAAM Institute Inc., Seattle, WA). Data for transport of Alexa-488-HDL to the SAC/ARC and the BC were fitted simultaneously to the model solutions (see Appendices A and B) using the relative data weighting option. Based on the covariance matrix of estimated parameters it is possible to generate sets of random kinetic parameters, which follow a normal distribution \( N(\mu, \Sigma) \) according to Equations 1.

\[
\phi(\mathbf{x}) = \mathbf{p} \cdot \exp \left( -\frac{1}{2} (\mathbf{x} - \mathbf{a})^T \mathbf{B}^{-1} (\mathbf{x} - \mathbf{a}) \right) \quad (\text{Eq. 1})
\]

Here, \( \mathbf{a} \) is the vector of actuarial expectations of the kinetic parameters (i.e. elements of \( \mathbf{a} \) are the parameters estimated by the non-linear regression), and \( \mathbf{B} \) is the inverse of the symmetrical covariance matrix. The kinetic parameters \( M \) and \( h_2 - h_3 \) form the vector \( \mathbf{x} \). Employing a Cholesky-decomposition \( B = D^T \cdot D \) and by replacing \( \mathbf{a} = D \cdot (\mathbf{x} - \mathbf{a}) \), it is possible to obtain random vectors of kinetic parameters \( \mathbf{z} \) from normal distributed elements \( u_i \) of the vector \( \mathbf{a} \) after back-transformation according to Equations 2.

\[
\hat{\mathbf{x}} = D^{-1} \cdot \mathbf{a} + \mathbf{u} \quad (\text{Eq. 2})
\]

Random vectors are generated using a Monte Carlo simulation as described (16). A program was written in Fortran code, which allows one to perform a Monte Carlo simulation of the obtained model solutions based on the sets of simulated kinetic parameters (16, 17).

**RESULTS**

**Modeling Supports Sequential Transport of Fluorescent HDL in Polarized HepG2 Cells**—Previously we could show that about 50–80% of initially bound Alexa-488-HDL recycle rapidly to the basolateral membrane of polarized HepG2 cells (9). Some fluorescent HDL was transported to the SAC/ARC whereas, with a lag-time, Alexa-488-HDL was also found in the BC (see Introduction). To model intrahepatic transport of HDL based on this kinetic data two assumptions have been made: (i) internalized fluorescent HDL was targeted from the basolateral electron microscope to SE from where rapid recycling occurred, (ii) some HDL escaping the recycling pathway was transcytosed to the BC and/or targeted to the SAC/ARC. From the data it could be concluded that transport of HDL from basolateral SE to the SAC/ARC and the BC, respectively, occurs either sequentially, i.e. HDL traverses the SAC/ARC before being targeted to the BC, or in parallel with compartments. To distinguish between these possibilities two models were derived in the following paragraph and fitted to the experimental time courses as described under “Experimental Procedures.” Transport of HDL to LE/LYS was not taken into account for comparison of the sequential and parallel transport models but was included later (see below). Because of the low number of data points in the measured transport kinetics of HDL and some uncertainty in the definition of various endosome populations in hepatic cells, a realistic trafficking model should contain as few kinetic parameters as possible (18).

(a) Sequential Model of Transcytosis—The sequential model

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shown in Fig. 1A gives the following system of differential equations with basolateral membrane (c₁), SE (c₂), SAC/ARC (c₃), and BC (c₄) in Equations 3–6.

\[
\frac{dc_1}{dt} = -k_1 \cdot c_1 + k_{-1} \cdot c_2 \\
\frac{dc_2}{dt} = k_1 \cdot c_1 - (k_{-1} + k_2) \cdot c_2 \\
\frac{dc_3}{dt} = k_2 \cdot c_2 - k_3 \cdot c_3 \\
\frac{dc_4}{dt} = k_3 \cdot c_3
\]

(Eq. 3)  
(Eq. 4)  
(Eq. 5)  
(Eq. 6)

The rapid equilibrium approximation (REA), which makes the assumption that uptake and recycling of HDL is much faster than export of HDL from SE to the SAC/ARC was applied (19). This gives Equations 7 and 8.

\[
c_2 = \frac{k_1}{c_1} \cdot k_{-1} = q \\
c_1 = \frac{k_{-1} \cdot c_2}{k_1}
\]

(Eq. 7)  
(Eq. 8)

When c₁+c₂ = M equals the amount of HDL in the basolateral membrane, and SE after an initial short time span c₁(≥0) and c₂(≥0) can be calculated with Equation 9,

\[
M = c_1 + k_{-1} \cdot c_2 = \frac{k_1}{k_{-1}} \cdot c_2
\]

(Eq. 9)

to Equation 10.

\[
c_1(≥0) = \frac{M \cdot k_1}{k_{-1} + k_1} \quad \text{and} \quad c_2(≥0) = \frac{M \cdot k_{-1}}{k_{-1} + k_1}
\]

(Eq. 10)

Because of the rapid equilibration between c₁ and c₂ and using Equation 8 (left) one will obtain Equation 11.

\[
\frac{dc_1 + c_2}{dt} = \frac{d}{dt}\left(\frac{k_{-1} \cdot c_2 + c_1}{k_1}\right) = \frac{d}{dt}\left(\frac{k_{-1} + k_1}{k_1} \cdot c_2\right) = -k_2 \cdot c_2
\]

(Eq. 11)

The differential Equation 4 for c₂(t) will therefore be as shown in Equation 12.

\[
\frac{dc_2}{dt} = \frac{k_2 \cdot c_2}{k_{-1} + k_1} = \frac{q \cdot k_2}{1 + q} \cdot c_2
\]

(Eq. 12)

The differential equation system consisting of Equations 5, 6, and 12 was solved with the initial conditions c₂(0) = (M · q)/(1 + q), c₃(0) = c₄(0) = 0 as described in “Appendix A.”

(b) Parallel Model of Transcytosis—The parallel model shown in Fig. 1B gives the following system of differential equations (Equations 13–15) in addition to Equation 3 with the same notation of compartments as described above.

\[
\frac{dc_2}{dt} = k_1 \cdot c_1 - (k_{-1} + k_2 + k_3) \cdot c_2
\]

(Eq. 13)

\[
\frac{dc_3}{dt} = k_2 \cdot c_2
\]

(Eq. 14)

\[
\frac{dc_4}{dt} = k_3 \cdot c_2
\]

(Eq. 15)

Because of the rapid equilibration between c₁ and c₂ as described in Equations 3 and 7 to 10, we obtain Equation 16.

\[
\frac{dc_2}{dt} = -k_1 \cdot k_2 \cdot c_2 = \frac{q \cdot k_2}{1 + q} \cdot c_2
\]

(Eq. 16)

The system consisting of Equations 14–16 was solved as described in “Appendix A.” Simultaneous fitting to all measured
compartment regression using SAAM II (SAAM Institute Inc., Seattle, WA) have a much lower standard deviation and coefficient of variance than those obtained for the parallel model (Fig. 2, C–F). This further argues for the sequential model but against the parallel model. Together, these results suggest that transport of fluorescent HDL from the basolateral membrane to the BC occurs by transit through the SAC/ARC but not in parallel to both compartments.

Validation of the Rapid Equilibrium Approach for HDL Recycling from Sorting Endosomes—It is important to validate the suitability of the REA for modeling HDL transport in hepatic cells. This can be done by comparing the solutions for the sequential model using the REA with those obtained from the original system without any system simplifying assumptions (called the complete sequential model). The differential equation system for the complete sequential model was solved (see Equations 3–6 and “Appendix B”), and the time course was simulated (Fig. 3). The internalization and recycling rates \( k_1 \) and \( k_{-1} \) were obtained from the literature: As shown for various cell types half-times of uptake from the plasma membrane and rapid recycling from SE are in the order of \( t_{1/2} = 2 \text{ min} \) and \( t_{1/2} = 1 \text{ min} \), respectively (20). Thus, for comparing both model solutions, the rates were set to \( k_1 = 0.35 \text{ min}^{-1} \) and \( k_{-1} = 0.7 \text{ min}^{-1} \). It is important to note that these values satisfy the condition \( q = 0.5 \) from the REA used for fitting the sequential model with REA to the data (see Fig. 1 and above). In Fig. 3A it is demonstrated that the time course for simulated fluorescence of Alexa-488-HDL in SE using the REA differs only for the first 2 min from the simulated time course of the complete sequential model in the SE. Using the complete sequential model a rapid initial rise for the simulated kinetics of HDL transport to SE was found (light gray line in Fig. 3A). After this short time span the time course of HDL transport from SE is
very well approximated by the sequential model using the REA (black line in Fig. 3A). This is further validated by the phase plot showing fluorescence of Alexa-488-HDL in the basolateral membrane (dark gray line) versus both solutions for the SE compartment (cSE(t)) (Fig. 3B) (19). This plot gives approximately a straight line for the REA (black line), which closely matches the phase curve for the complete sequential model (gray line) for values of fractional fluorescence of Alexa-488-HDL in the basolateral membrane of up to 0.05. Only for larger values of cSE(t) both curves deviate, which reflects the first 2 min of simulation (compare Fig. 3A).

Next the simulated time courses of fractional fluorescence of Alexa-488-HDL in the SAC/ARC and BC, respectively, were compared for the sequential model with REA and the complete sequential model (Fig. 3, C and D). Employing the REA has almost no impact on the simulated kinetics of HDL transport to both compartments. In other words, using the REA will only minimally affect the estimated kinetic parameters obtained from the regression of the model to the experimental time courses of fluorescence of Alexa-488-HDL in the SAC/ARC and BC, respectively. It can be concluded that the REA is a valid assumption for modeling HDL transport in hepatocellular too.

Previously we determined a rate constant of \( k_0 = 0.818 \text{ min}^{-1} \) at \( 4^\circ C \) and dissociation \( k_- = 0.048 \text{ min}^{-1} \) at \( 4^\circ C \) of HDL on HepG2 cells have been measured previously (22). A numerical simulation of the complete sequential model (see "Appendix B" and above) with an additional binding/release step for HDL on HepG2 cells using these rate constants gives a half-time for the release process of \( t_{1/2} = 8 \text{ min} \) and a plateau value of cell-associated fluorescence of labeled HDL of 70% of initial (not shown). Here, the assumption was made that initially all fluorescent HDL is bound to the cell surface, i.e., in the basolateral plasma membrane (c(0), see Equation 3) mimicking the experimental conditions from our previous study (9). Since in our pulse experiment at \( 37^\circ C \), association and dissociation might be faster than at \( 4^\circ C \), the observed half-time of release of Alexa-488-HDL from HepG2 cells in our previous experiments of \( t_{1/2} = 6.9 \text{ min} \) is in good agreement with those binding values (9, 22). This indicates that the rate-limiting steps for fluorescence loss of Alexa-488-HDL in HepG2 cells as measured previously are binding and dissociation rather than recycling of HDL, which is assumed to be much faster (see Fig. 3 and Ref. 20).

Apical Endocytosis of Lysosomal Markers in Living HepG2 Cells—Apical endocytosis of transfected proteins has been shown previously in polarized hepatic cells (18, 23). It was suggested that canalicular endocytosis targets proteins directly to LE/LYS (23). Rh-dextran is a marker for LE/LYS as well as for the BC, and fluorescent HDL colocalized with Rh-dextran after prolonged chase (9). To test the hypothesis that apical endocytosis leads to LE/LYS in polarized hepatic cells, BC-associated fluorescence of this lysosomal marker was next quantified from images of Rh-dextran-labeled HepG2 cells after various chase times. There is a time-dependent decrease of fluorescence of Rh-dextran in the BC compartment that fits a mono-exponential function. The half-time of this kinetics is \( k = 0.016 \text{ min}^{-1} \) \( (t_{1/2} = 43.3 \text{ min}) \) which resembles that high molecular mass dextrans cannot permeate through the tight junctions of hepatic cells for prolonged chase times (24). Moreover, normalization of the data to total cellular fluorescence accounts for any loss of Rh-dextran from cells. The results suggest that Rh-dextran becomes slowly internalized from the BC and delivered to LE/LYS.

Alexa-488-HDL also colocalized with fluorescent ASOR in LE/LYS after prolonged chase (9). About 2–5% of radioactively labeled ASOR perfused into rat liver is secreted into the bile within a 30-min chase (25–27). In primate liver about 7% of perfused ASOR become released into the bile after a 47-min chase (28). Thus, although most ASOR is degraded in LE/LYS, a small portion of basolaterally internalized ASOR should be delivered to the canalicular membrane for subsequent biliary secretion. In HepG2 cells, ASOR is rapidly sorted from Tfn in SE and delivered to LE/LYS (29). Previously, we demonstrated that fluorescent ASOR is transported to LE/LYS in a time course of 60 min (12). As shown in Fig. 4, B–H some fluorescent TexRed-ASOR is transported to the BC of polarized HepG2 cells within 20–30 min chase at \( 37^\circ C \). This likely resembles biliary secretion of ASOR as found in hepatocytes. Importantly, after prolonged chase of about 1 h BC-associated fluorescence of TexRed-ASOR decreases (Fig. 4, B and F–H). This clearly indicates that also TexRed-ASOR becomes internalized from the BC. It should be emphasized that TexRed-ASOR was found in the BC only after prolonged chase in the absence of the probe. If TexRed-ASOR would have direct access to the BC lumen, BC should be stained right after labeling with the probe. This was not observed. Thus, permeation of TexRed-ASOR through the tight junctions of HepG2 cells during the time course of the experiments can be excluded. The decrease of...
BC-associated fluorescence found between 30 and 70 min of total chase time reflects therefore most likely apical internalization of TexRed-ASOR. In conclusion, these results show that two lysosomal markers, which colocalize with Alexa-488-HDL after prolonged chase become slowly internalized from the BC of polarized HepG2 cells. It supports that one endocytic pathway from the BC leads to LE/lys.

Direct Visualization of Canalicular Endocytosis by Time-lapse Imaging of DiIC12—To obtain conclusive evidence for apical endocytosis would require to observe directly the process of vesicle budding from the canalicular membrane. Because of their superb fluorescence properties including high quantum yield DiI analogs are very suitable for time-lapse experiments. Cells were pulse-labeled with DiIC12 and imaged with low light level acquisition at a temperature-controlled microscope stage of a wide-field microscope maintained at 36 ± 1 °C. Representative time-lapse sequences are shown in Fig. 5A: After starting the video sequence a vesicle (blue arrowhead) moved from the basolateral membrane to the subapical region underneath the BC in a time course of 6 min (arrow). At the BC some endocytic vesicles were found which stayed associated with the apical pole forming a subapical vesicle pool (red arrowheads). The vesicle derived from the basolateral membrane was transported to this subapical region and associated with the apically derived vesicle pool (panels 5 and 6). After 7 min, apical vesicles were expelled into the cytoplasm (red arrowheads), and new vesicles formed at the BC (panel 8, red arrowheads). These time-lapse results indicate that endocytotic vesicles form at both plasma membrane domains.

To monitor directly the process of vesicle budding from the canalicular membrane cells were labeled for 1 h with DiIC12, washed and time lapse sequences were acquired every 10 s at a wide-field microscope. Fig. 5B shows vesicle trafficking from the canalicular membrane of those cells with every panel corresponding to a 10-s time interval. Repeated vesicle formation was found at three positions of the canalicular membrane (indicated by red, green, and blue arrowheads, respectively). Those vesicles occasionally stayed associated with the canalicular membrane for some time before release (panels 27–40, blue arrowhead). A typical time course for vesicle budding from the canalicular membrane was 30–50 s but also long-time association of vesicles with the donor membrane for more than 2 min was found. In panel 14 vesicular structures could be observed in the BC lumen (blue arrows). As shown in Fig. 5C, two vesicle-like structures were found in the BC lumen during the time of observation of 5 min (blue arrows). In the same time course, seven vesicles were found to bud from the BC toward the cytoplasm (see Supplemental Data). From these observations the following can be concluded (see scheme in Fig. 5D): (i) endocytosis of lipid analogs occurs from preferred sites or “spots” at the canalicular membrane, (ii) internalized vesicles can stay associated with the BC for prolonged time, and (iii) vesicles seemed to be released also to the BC lumen of HepG2 cells, which could well be a remnant of biliary lipid secretion (see “Discussion”).

From time-lapse sequences like those shown in Fig. 5, A–D fluorescence of DiIC12 in the BC was quantified. BC-associated fluorescence of DiIC12 showed a rapid initial rise for about 5 min followed by decreasing fluorescence intensity up to a 30-min chase (Fig. 5E, gray symbols). The time-dependent decrease of BC-associated fluorescence of DiIC12 could be well fitted by a monoexponential function giving a half-time of 43.3 min (Fig. 5E, straight line). These results suggest that after rapid, probably non-vesicular transport of DiIC12 to the BC the lipid analog becomes slowly internalized by apical endocytosis.

Complete Mathematical Description of Measured Kinetics of HDL Transport in HepG2 Cells—Next, the sequential transport model was extended to include lysosomal targeting of...
HDL. Previously, we determined transport kinetics of Alexa-488-HDL to LE/lymph stained with Rh-dextran (9). It is well known that basolateral SE transport certain ligands and fluid phase markers to LE/LYS in polarized (hepatic) cells (29–31). Transport to LE/LYS occurs from SE, which mature into late endosomes by losing their fusion competence to receive new internalized material (31, 32). To model this process, unidirectional transport was included from basolateral SE to LE/LYS in the sequential transport model using the REA (rate constant $k_4$). Previously, we found that BC-associated fluorescence of Alexa-488-HDL decreases after prolonged chase in polarized HepG2 cells. This was found when cells were labeled for 10 min with fluorescence HDL and chased for 30 and 60 min at 37 °C (see Fig. 6E) (9). An analog targeting pathway from the BC has been described previously for transport of transcytotic protein markers in polarized WIF-B cells (18). Experimental evidence has been provided that apical and basolateral endocytic pathways merge in LE/LYS in polarized (hepatic) cells (23, 33, 34). To include apical endocytosis in a HDL transport model requires the assumption that fluorescent HDL is transported to LE/LYS from both plasma membrane domains (7, 34, 35). This was included by assuming a rate constant ($k_6$), which accounts for degradation of HDL in the lysosomal compartments. According to the scheme shown in Fig. 6A one obtains the following differential equation system to describe HDL transport by this complete model named extended sequential transport model (ESTM). The model includes transport from the basolateral membrane to SE ($c_2(t)$)
using the REA (Equations 7–10) and from this compartment either to the SAC/ARC ($c_3(t)$) followed by export to the BC ($c_4(t)$) or to the LE/LYS ($c_5(t)$). This gives Equations 17–20.

$$\frac{dc_2}{dt} = \frac{-q \cdot (k_2 + k_4)}{q + 1} \cdot c_2$$  
(Eq. 17)

$$\frac{dc_3}{dt} = k_2 \cdot c_2 - k_3 \cdot c_3$$  
(Eq. 18)

$$\frac{dc_4}{dt} = k_3 \cdot c_3 - k_5 \cdot c_4$$  
(Eq. 19)

$$\frac{dc_5}{dt} = k_4 \cdot c_2 + k_5 \cdot c_4 - k_6 \cdot c_5$$  
(Eq. 20)

Eigenvalues and eigenvectors of the corresponding coefficient matrix were calculated, and the system was solved for the initial conditions $c_2(0) = (M \cdot q)/(1 + q)$, $c_3(0) = 0$ or $c_3(0) = L$, $c_4(0) = c_5(0) = 0$, yielding solutions for all compartments as described in “Appendix A.”

To compare this model with the experimental data requires more than two time courses to be included in the fitting procedure (see Fig. 1). Therefore, the measured kinetics for transport of HDL to LE/LYS were included (Fig. 6D) (9). Previously obtained data for transport of fluorescent HDL to the BC in cells pulse-labeled for 10 min with Alexa-488-HDL was additionally used for the fitting procedure. The latter kinetics can be compared with the model only, if the ESTM would be additionally solved for the conditions of a non-zero fraction of fluorescent HDL, $L$, already in the SAC/ARC. To fit the 10-min pulse data to the model, in the ESTM, the initial fluorescence of Alexa-488-HDL in the BC was set to zero. Accordingly, this model solution accounts for the long pulse experiment where some HDL is already transported to the SAC/ARC at beginning of the chase. The second assumption of an empty BC is only approximately valid but reduces the number of parameters required to fit to the data. The uptake/recycling ratio, $q$, was set to $q = 0.5$ (see Fig. 1), while the fraction of fluorescent HDL in the SAC/ARC at time point $t = 0$ for the 10-min pulse experiment, $L$, was set to $L = 0.05$. The ESTM was fitted next simultaneously to the experimental data for the 1-min pulse kinetics in the SAC/ARC (B), the BC (C), and the LE/LYS (D) very well. Moreover, the model can account for the 10-min pulse kinetics of the BC (E) with a decrease in BC-associated fluorescence of labeled HDL for prolonged chase. The ESTM

FIG. 6. ESTM for fluorescent HDL with lysosomal targeting. A, sequential model shown in Fig. 1A was extended to include transport of fluorescent HDL from SE to LE/LYS giving the ESTM. This is based on previous measurements for HDL transport to LE/LYS in polarized HepG2 cells (9). Solutions for this model (see “Appendix A”) were fitted simultaneously to the experimental time courses for Alexa-488-HDL transport to the SAC/ARC (B), the BC (C), and to LE/LYS (D) from a 1-min pulse experiment as well as to the BC from a 10-min pulse experiment (E) using SAAM software. The model was fitted simultaneously to all compartments with a fixed ratio $k_2/k_3 = q = 0.5$ using the REA, leaving $q$ and $M$ as free parameters to be estimated by the regression. Gray symbols represent data while straight lines show the fitting result. The obtained parameters including S.D. estimated by the model are shown (F).
fitted with SAAM II gives kinetic parameters with low standard deviation (Fig. 6 G). Indeed, the standard deviation is lower than that of parameters fitted with the sequential model shown in Fig. 1. The ESTM suggests that fluorescent HDL is slowly ($t_{1/2} = 28.7$ min) internalized from the BC and targeted to LE/lys for degradation. These results clearly show that it is possible to derive rate constants for complex transport processes from multiple time courses obtained from quantitative fluorescence imaging data.

**Statistical Assessment of the Extended Sequential HDL Transport Model**—It is important to determine how the errors for different fitted parameters affect the overall result of the regression procedure. This can be done by simulating the model with randomly generated kinetic parameter sets based on the covariance matrix as determined using SAAM II (16, 17). Random vectors were generated for the fitted parameters $M$, and $k_2$, $k_6$ according to a 6-dimensional normal distribution (see “Experimental Procedures”). Based on those parameter sets time courses for the various compartments were simulated using the ESTM for HDL in hepatic cells. In Fig. 7 the simulated time course for Alexa-488-HDL fluorescence in the individual compartments of polarized HepG2 cells is shown. Simulated kinetic parameters were obtained by 500 iterations of the Monte Carlo algorithm, and the time course gives the mean ± S.D. (compare Fig. 6 F). C–H, covariance ellipses estimated from Monte Carlo-simulated kinetic parameters. C–E, parameters $M$ representing the initial fraction of HDL in the basolateral membrane were plotted against the kinetic parameters $k_2$ (C), $k_3$ (D), and $k_4$ (E) as obtained from the Monte Carlo simulation. F–H, Monte Carlo-simulated kinetic parameters $k_2$ were plotted against $k_3$ (F) and $k_4$ (G), respectively, while parameters $k_3$ were plotted versus $k_4$ (H). See text for further explanations.
covariance ellipses as obtained from 500 iterations of the Monte Carlo algorithm are shown for various combinations of simulated parameters. All estimated parameters show a reasonably low width of distribution. This is despite the low number of data points of individual transport kinetics measured previously (9). It underlines how fitting a theoretical model to data can be improved by including multiple time courses from (i) various compartments and (ii) various pulse-chase regimes. Important conclusions can be drawn from the covariance analysis: From the distribution of simulated parameters, it can be seen that \( k_1 \) and \( k_2 \) which model transport of fluorescent HDL from basolateral SE to the SAC/ARC and from the SAC/ARC to the BC, respectively, increase when the value of the initial fraction \( M \) increases, i.e. both parameters are positively correlated with \( M \) (Fig. 7, C and D). In contrast, the parameter \( k_4 \) which models transport of Alexa-488-HDL from SE to LE/LYS, does not have strong correlation with \( M \) (Fig. 7E). Furthermore, the parameter \( k_3 \) increases when \( k_4 \) increases, i.e. both parameters are positively correlated (Fig. 7F). There seems to be no significant correlation between the fitted parameters \( k_2 \) and \( k_3 \) or \( k_3 \) and \( k_4 \), respectively (Fig. 7, G and H). Monte Carlo simulation of a given model based on fitted parameters is very suitable to determine the measurement error associated with experimental designs but also to estimate the degree of correlation of the parameter set (16).

**Quantitative Assessment of Free Sterol Sorting from HDL by SR-BI**—By fluorescence recovery after photobleaching (FRAP) we showed recently, that DHE is rapidly transported to the BC by an ATP-independent process with a rate constant of \( k_+ = 0.448 \text{ min}^{-1} \) (10). Transport in the basolateral direction was slower but occurred also in cells depleted of metabolic energy (rate constant \( k_- = 0.129 \text{ min}^{-1} \)). In an independent set of experiments we showed that this rapid non-vesicular transport is also responsible for sorting of DHE from fluorescent HDL at the basolateral membrane of HepG2 cells by a SR-BI mediated process (see Introduction) (9). By comparing the BC-associated fluorescence intensity of DHE derived from HDL in normal and ATP-depleted HepG2 cells, we estimated that less than 10% of initial DHE in HDL reach the canalicular membrane as constituent of the HDL particles (see Fig. 5G in Ref. 9). Here, it is shown that this estimation can be supported and even refined using the ESTM for HDL in hepatic cells. DHE in the BC, as measured previously, was replotted as function of time. In those experiments cells were labeled for 1 min at 37 °C either with DHE in a cyclodextrin complex (CD, Fig. 8A, gray symbols) or with Alexa-488-HDL containing DHE (Fig. 8B, gray symbols), washed, and imaged. Fluorescence of DHE in the BC was measured and normalized to initial BC-associated DHE fluorescence (right after labeling). After starting the experiments (first image) BC were brightly labeled irrespective of the labeling method (see above and Refs. 9 and 10). In cells, where DHE was applied by labeling with DHE bound to CD, BC-associated fluorescence of DHE increased by about 13% after 30 min of chase (Fig. 8A). This can be well approximated by a mono-exponential function with \( k_+ = 1.139 \text{ min}^{-1} \) corresponding to a half-time of \( t_{1/2} = 36.5 \text{ s} \) (Fig. 8A, straight line). This is faster than the rate constant of BC-directed transport of DHE measured previously by FRAP in HepG2 cells (see above) (10). Fluorescence recovery kinetics depends on the size of the bleach spot as well as on the time required for bleaching. In fact, in models for determining apparent diffusion rates from FRAP experiments, it is assumed that the bleaching process is much faster than the recovery process, a condition not fulfilled in our previous FRAP experiments (10, 36). This can result in a gross underestimation of diffusion kinetics when the bleaching time is extended (37). It is presumed that the rate constant measured by FRAP strongly underestimates the transport velocity of DHE to the BC and that the rate constant of \( k_+ = 1.139 \text{ min}^{-1} \) given above more accurately describes the transport process. Using the same quantification protocol it is found that fluorescence of DHE derived from HDL raised by about 17% in the BC after a 30–60 min chase (Fig. 8B). Despite the rather large measurement error in these experiments, it can be concluded that the time-dependent increase of BC-associated fluorescence of DHE derived from HDL is slightly higher than that of DHE derived from CD (compare 13 and 17% increase for DHE derived from CD and HDL, respectively). Using the ESTM the fraction of DHE transported to the BC as part of HDL can be estimated from this data: a mono-exponential function with fixed new rate constant of non-vesicular sterol transport to the BC of \( k_+ = 1.139 \text{ min}^{-1} \) (see above) was added to the solution of the ESTM for the BC with fixed kinetic parameters \( k_2 \) and \( q \) (see Figs. 6 and 7 and “Appendix A,”...
Fig. 9. Summary of modeling results for transport of HDL and its free sterol in hepatic cells. HDL binds to SR-BI at the basolateral membrane of polarized hepatic cells followed by rapid release of free cholesterol (FC) from HDL and uptake of HDL by SR-BI-mediated endocytosis. Internalized HDL is transported to basolateral SE with a half-time of $t_{1/2} = 2$ min from which endosome most HDL rapidly recycles with a half-time of $t_{1/2} = 1$ min. Both half-times are derived from the literature (indicated by * and were used for the rapid equilibrium approach (REA, see Fig. 3) (20, 45). From the basolateral SE some HDL enters the transcytotic pathway being delivered to the SAC/ARC ($t_{1/2} = 4.7$ min) followed by export to the BC ($t_{1/2} = 10.9$ min). Basolateral SE gradually lose their fusion competence and become with time LE/LYS containing a small amount of HDL ($t_{1/2} = 25.5$ min) (31, 32). Because of continuous apical endocytosis HDL in the BC becomes internalized and delivered to LE/LYS also from the apical membrane ($t_{1/2} = 28.7$ min) (see results on apical endocytosis in HepG2 cells shown in Figs. 4 and 5) (23, 34). HDL in LE/LYS is finally degraded ($t_{1/2} = 8.4$ min) (7, 35). Most FC released from HDL is shuttled to the BC by rapid non-vesicular transport (94% of total in BC), while only 6% of total FC in the BC is co-transported with HDL (9, 10). Half-times for HDL transport in the figure represent the range given by a 95% confidence interval, while half-times given above in brackets are the mean values as obtained by fitting the model to data (see Fig. 6). Numbers in brackets in the figure express the percentage of BC-directed transport of FC according to the fit shown in Fig. 8. Half-times for free sterol transport to the BC ($t_{1/2}^f$) and from the BC ($t_{1/2}^i$) are given for non-vesicular transport (dotted line) (10).

Equation A8). This gives a function where only the amplitudes $A$ (for non-vesicular sterol transport) and $M$ (for vesicular sterol transport with HDL) have to be estimated by a non-linear regression (Fig. 8B). The result of this fit are shown in Fig. 8B (straight black line labeled $\sum$). For comparison the mono-exponential part of this combined function with the fitted parameter $A$ is shown as dashed line in panel B (labeled released DHE). The lower straight line represents the portion of DHE in the BC co-transported with HDL (labeled DHE bound to HDL) according to the fit obtained with the combined function (see above). It can be concluded that the maximal amount of DHE transported to the canalicular membrane as constituent of HDL is 6.4% of total DHE transported to the BC. For the BC but also for all other compartments (i.e. the SE, SAC/ARC, and LE/LYS, not shown) the fraction of DHE co-transported with HDL is therefore too low to be detected by fluorescence microscopy (compare Ref. 9). This makes mathematical modeling based on HDL transport data a very useful alternative. To model sterol transport vesicular transcytosis of DHE was not included in the model. We found previously that DHE traverses HepG2 cells in both directions in vesicles, however, once associated with the SAC/ARC, sterol exchange between the plasma membrane domains in vesicles was very low. Thus, to neglect vesicular transcytosis of DHE from the basolateral to the canalicular membrane in the above-described analysis is a valid assumption. In conclusion, the ESTM with estimated kinetic parameters for HDL transport can be employed to quantify SR-BI-mediated free sterol sorting from HDL in hepatic cells.

**DISCUSSION**

The proposed transport model for HDL in polarized HepG2 cells developed in this study combines several aspects of endocytosis, recycling and transcytosis of various ligands in hepatic cells (Fig. 9). Indeed, basolateral uptake, transit through SE and targeting to the apical membrane via subapical recycling endosomes has been suggested to be the underlying transport scheme for transcytosis of various proteins in hepatocytes (30). Most apical proteins are shuttled from the Golgi after biosynthesis first to the basolateral membrane before they get targeted to the BC by transcytosis (38–40). For example, 5’-nucleotidase (5’-NT), aminopeptidase N (APN), polymeric immunoglobulin A receptor (pIgA-R), and dipeptidyl peptidase IV (DPPIV) traffic all from the basolateral membrane through the SAC/ARC to the BC in polarized WIF-B cells and in primary hepatocytes (30, 40). Radioactively labeled HDL has been shown to redistribute to the same intracellular compartments like 5’-NT in vivo (41). SE have been implicated in rapid recycling of membrane constituents in various studies (20, 21, 42–44). Sheff et al. (21, 42) could demonstrate that the perinuclear endocytic recycling compartment (ERC) is not obligatory accounting only for 24% of recycling of Tf, while the remaining 76% recycle from SE in Chinese hamster ovary (CHO) cells (42). The authors could confirm the existence of these two recycling pathways in polarized MDCK cells (21). Rapid internalization and recycling of the fluid-phase marker inulin was found in primary hepatocytes with $t_{1/2} \leq 2$ min for both processes. About 2% of internalized inulin was secreted into the bile (45). It is possible that some fluorescent HDL recycles also from the SAC/ARC perhaps together with a portion of fluorescent Tf. However, the kinetic analysis shown in Figs. 1–6 based on transport data measured previously clearly suggests that most recycling of HDL occurs from SE prior to targeting of the lipoprotein to the SAC/ARC. From basolateral SE proteins destined for degradation become targeted to LE/LYS by maturation of SE as shown for HepG2 cells and other cell types (29, 31, 32, 46).

Apical endocytosis has been predicted for hepatic cells based on calculations of membrane area exchanged by transcytosis and biliary release (47, 48). Internalization of proteins, fluid
phase markers and fluorescent lipids including sterol from the canicular membrane has been reported (10, 12, 13, 18, 34). However, derivation of rate constants for canicular endocytosis was not possible because of the difficult experimental setup in labeling specifically the BC. Here, it is shown that mathematical modeling allows one to predict rate constants for hepatic apical endocytosis based on commonly employed transcytosis assays (9, 30). Importantly, it could be shown in this paper that the half-time for apical internalization of fluorescent HDL determined by mathematical modeling of complex trafficking data ($t_{1/2} = 28.7$ min, see Fig. 6) closely matches the apical endocytosis rate measured directly for DiIC12 in living HepG2 cells ($t_{1/2} = 25.5$ min, see Fig. 5). The model shown here does allow one to derive rate constants for apical endocytosis of other proteins that have been shown to become internalized from the BC like APN and 5'-NT as soon as more quantitative trafficking data from the canicular membrane becomes available (18, 23). The ESTM shown in Fig. 6 and applied to hepatic HDL transport is in accordance with the hypothesis that the SAC/ARC is not traversed by proteins internalized from the canicular membrane. This has been shown for various apical protein markers but was questioned in other protein trafficking studies (18, 23, 49). Here it is shown, that lysosomal markers, Rh-dextran and TexRed-ASOR become internalized from the BC (see Fig. 4). Importantly, no colocalization of those markers with fluorescent Tf in the SAC/ARC was found after prolonged chase when BC-associated fluorescence of Rh-dextran and TexRed-ASOR decreased (not shown). This supports that one endocytic pathway leads from the BC to LE/LYS bypassing the SAC/ARC as reported (18, 23). Based on the modeling results, it is suggested that this pathway is also followed by a portion of fluorescent HDL after its targeting to the canicular membrane in HepG2 cells (see Fig. 6). Importantly, it was found that SR-BI, which mediates uptake and intrahepatic transport of HDL, is expressed at the canicular membrane in vivo and in hepatocyte couplets (5, 50, 51). Whether SR-BI mediates also apical internalization and lysosomal targeting of a portion of HDL in hepatic cells remains to be clarified.

Time-lapse sequences demonstrate internalization of DiI anologs from the canicular membrane (see Fig. 5). Strikingly, release of DiI-containing vesicles into the canicular lumen was also observed (see Fig. 5C). This could be a remnant of biliary lipid secretion in polarized HepG2 cells. Indeed, in hepatocyte couplets vesicle secretion into the BC lumen was demonstrated by light scattering spectroscopy as also found in the liver (52, 53). Together with our previous studies it underlines the suitability of HepG2 cells for investigations of hepatic transport (9, 12). DiIC12 is a marker for recycling endosomes but not for LE/LYS as shown for non-polarized cells (15). When HepG2 cells were colabeled with DiIC12 and Alexa-488-Tf, both probes showed almost complete overlap in vesicles underneath the BC indicating transport to the SAC/ARC (not shown). This suggests recycling of vesicles containing DiIC12 between the BC and the SAC/ARC. Similar results were found previously for fluorescent PC and sphingolipids as well as for DHE (10, 12, 13). None of the studied lipids colocalized with lysosomal markers.

ATP binding cassette (ABC) transporters implicated in bile secretion were shown to recycle between the SAC/ARC and the BC as well (49, 54). However, in contrast to 5'-NT, DPPIV or APN various ABC proteins are targeted directly from the trans-Golgi network to the BC, i.e. they don’t follow the transcytotic route (55–57). Another important difference between the former apical proteins and ABC transporters is that 5'-NT, DPPIV, and APN but not ABC transporters are released into the bile. Similarly, apoprotein AI and AII, the major apoproteins of HDL, were found to be secreted into the bile after administration of labeled HDL to perfused rat livers (58). In the bile these apoproteins prevent crystallization of cholesterol (59). Thus, it can be speculated that a part of proteins released into the bile or the BC lumen in the cell culture system after transcytosis from the basolateral membrane become retrieved from the BC and targeted to LE/LYS for degradation. Proteins with transport function in the canicular membrane as well as bulk membrane lipid might recycle between the BC and the SAC/ARC in polarized hepatic cells. An apical recycling pathway might be important to maintain a constant canicular surface area (47, 48). Two apical endocytic pathways were also found in polarized Madin Darby Canine Kidney (MDCK) cells: plgA-R and Tf internalized from the apical membrane recycle toward the apical cell surface from an ARC (60, 61). Co-internalized plgA-R and fluorescent dextran become targeted from the apical membrane to SE where both markers segregate, i.e. plgA-R traffics along the transcytotic pathway while fluorescent dextran is delivered to LE/LYS (62). Markers for apical SE were also found underneath the BC in polarized hepatic cells (23, 63). It is also possible that apical recycling occurs from apical SE, which might be difficult to distinguish from the SAC/ARC in some hepatic cell types. Further studies are warranted to clarify the function and sub-compartmentalization of the SAC/ARC in hepatic cells.

The HDL transport model has been extended to estimate the extent of free sterol sorting from HDL at the basolateral cell surface of hepatic cells by SR-BI (see Fig. 8 and Ref. 9). It could be shown that only about 6% of free sterol are transported to the BC as constituent of HDL while the remaining 94% reach the canicular membrane by non-vesicular transport after release from the HDL particles (see Figs. 8 and 9). This modeling approach can be used to quantitatively assess sorting of other lipids than free sterol from HDL in future studies. Previously, we demonstrated that also fluorescent PC is transported to the BC by a non-vesicular pathway (12). Based on in vivo data by Portal et al. (64) we speculated that PC in HDL could follow a similar pathway as free sterol, i.e. release at the basolateral membrane by SR-BI and non-vesicular shuttling to the canicular membrane independent of the HDL apoprotein (12, 64). In line with this hypothesis is the observation that SR-BI mediates also the uptake of HDL phospholipids into cells (65–67). In cerebrovascular endothelial cells natural PC was selectively internalized from HDL by SR-BI (68). On the other hand, a slow and a fast exchanging component of HDL PC has been reported, and it is likely, that a large portion of PC will be co-transported with HDL apoprotein in hepatic cells (69). In further experiments measuring the kinetics of canicular enrichment of HDL PC in HepG2 cells, both pathways could be quantified using the model described in this paper. This will allow one to estimate the extent of phospholipid sorting from HDL mediated by SR-BI during intracellular transport of the lipoprotein particle.

In conclusion this study shows that the combination of mathematical modeling with quantitative fluorescence microscopy is a very suitable approach for dissecting complex trafficking schemes in living mammalian cells. This is especially important when sorting processes are investigated where one transport step cannot be isolated experimentally. Development of a kinetic model, which includes known, kinetically characterized transport steps can help to derive rate constants for the process under study. This approach is well known from metabolic analysis and should find broad application in membrane trafficking studies as well (19).
APPENDIX

Appendix A—Here, the solutions for the differential equation systems modeling transport of HDL in polarized HepG2 cells are given. Equations to be solved are indicated in parentheses. These were obtained by calculating the eigenvalues and eigenvectors of the corresponding coefficient matrix. They were used to fit the experimental data of Wustner et al. (9) to the SAC/ARC("c_0(t)""); to the BC (c_0(t)) and to the LE/LYS (c_0(t)) (9). The solution for SE is given by the expression c_0(t) for the sequential and parallel model, but was not used to fit the data.

(a) Sequential transport (Equations 5, 6, and 12).
\[ c_0(t) = \frac{M \cdot q}{1 + q} \cdot \exp \left( -\frac{k_2 \cdot q}{1 + q} \cdot t \right) \]  
(Eq. A1)
\[ c_0(t) = \frac{k_2 \cdot M \cdot q}{k_2 + k_3 \cdot q + k_4 \cdot q} \cdot \left( \exp \left( -\frac{k_2 \cdot q}{1 + q} \cdot t \right) - \exp(-k_3 \cdot t) \right) \]  
(Eq. A2)
\[ c_0(t) = \frac{M + \frac{k_2 \cdot M \cdot q}{k_2 + k_3 \cdot q + k_4 \cdot q} \cdot \left( \exp(-k_3 \cdot t) - \frac{k_3 \cdot (1 + q)}{k_2 \cdot q} \cdot \exp \left( -\frac{k_2 \cdot q}{1 + q} \cdot t \right) \right)}{k_3 + k_4} \]  
(Eq. A3)

(b) Parallel transport (Equations 14–16).
\[ c_0(t) = \frac{M \cdot q}{1 + q} \cdot \exp \left( -\frac{k_2 + k_3 \cdot q}{1 + q} \cdot t \right) \]  
(Eq. A4)
\[ c_0(t) = \frac{k_2 \cdot M}{k_2 + k_3} - \frac{k_3 \cdot M \cdot q}{k_2 + k_3} \cdot \exp \left( -\frac{k_2 + k_3 \cdot q}{1 + q} \cdot t \right) \]  
(Eq. A5)
\[ c_0(t) = \frac{k_2 \cdot M}{k_2 + k_3} \cdot \exp \left( -\frac{k_2 + k_3 \cdot q}{1 + q} \cdot t \right) \]  
(Eq. A6)

(c) Sequential transport from both plasma membrane domains (Equations 17–20),
\[ c_0(t) = \frac{(k_3 - k_5) \cdot (k_2 - k_5)}{k_5} \cdot b_1 \cdot \exp(-k_3 \cdot t) \]  
\[ - \frac{k_2 \cdot (a - k_3) \cdot (a - k_5)}{G} \cdot b_4 \cdot \exp(-a \cdot t) \]  
(Eq. A7)
\[ c_0(t) = -\frac{k_2 \cdot k_5 \cdot b_1 \cdot \exp(-k_3 \cdot t) - \frac{k_2 \cdot k_5 \cdot b_2 \cdot \exp(-k_3 \cdot t)}{G}}{G} \cdot b_4 \cdot \exp(-a \cdot t) \]  
(Eq. A8)
\[ c_0(t) = b_1 \cdot \exp(-k_3 \cdot t) + b_2 \cdot \exp(-k_3 \cdot t) + b_3 \cdot \exp(-k_5 \cdot t) \]  
\[ + b_4 \cdot \exp(-a \cdot t) \]  
(Eq. A9)

with
\[ a = \frac{(k_2 + k_3) \cdot q}{1 + q} \]  
(Eq. A10)
\[ G = k_2 \cdot k_3 \cdot k_5 \cdot k_4 \cdot (a - k_3) \cdot (a - k_5) \]  
(Eq. A11)
\[ b_1 = \frac{(a - k_3) \cdot (a - k_5)}{G \cdot (k_2 + k_3 \cdot k_5 - k_4)} \cdot b_4 \]  
(Eq. A12)
\[ b_2 = \frac{k_3 - k_5}{k_5 - k_6} \cdot b_1 + \frac{(a - k_3) \cdot k_2 \cdot k_5}{G \cdot (k_2 - k_6)} \cdot b_4 \]  
(Eq. A13)
\[ b_3 = - (b_1 + b_2) \]  
(Eq. A14)

To fit the 10-min pulse experiment, the system shown in Equations 17–20 was additionally solved for the initial conditions c_0(0) = (M \cdot q)/(1 + q), c_0(0) = L, c_0(0) = c_0(0) = 0 yielding for the BC (c_0(t)),
\[ c_0(t) = \frac{k_1 \cdot (k_5 - k_3) \cdot S \cdot \exp(-k_3 \cdot t) - N \cdot \exp(-a \cdot t)}{(k_3 - k_5) - (k_3 - k_5) \cdot \exp(-k_3 \cdot t) - \exp(-a \cdot t)} \]  
(Eq. A16)

with
\[ S = \frac{G \cdot k_2 \cdot k_4 \cdot (a - k_3) \cdot M \cdot q}{(G + k_2 \cdot k_3 \cdot k_4 \cdot (1 + q))} \]  
(Eq. A17)
\[ N = \frac{k_3 \cdot k_4 \cdot M \cdot q}{(G + k_2 \cdot k_3 \cdot k_4 \cdot (1 + q))} \]  
(Eq. A18)
\[ P = \frac{(k_3 - k_4)}{k_3} \cdot \left( \frac{k_3 \cdot k_4 \cdot (k_3 - k_5) \cdot S \cdot \exp(-k_3 \cdot t) + k_4 \cdot N}{(k_3 - k_5) - (k_3 - k_5) \cdot \exp(-k_3 \cdot t) - \exp(-a \cdot t)} \right) \]  
(Eq. A19)

Appendix B—To determine the validity of the REA for the analysis of hepatic HDL transport, the differential equation system describing the sequential model without simplification was solved and compared with the sequential model with REA (see Equations 3–6 and "Appendix A"). The solutions for the basolateral membrane (c_0(t)), the SE (c_0(t)), the SAC/ARC (c_0(t)) and the BC (c_0(t)) were obtained by calculating the eigenvalues and eigenvectors of the corresponding coefficient matrix for the initial conditions c_0(0) = M, c_0(0) = c_0(0) = c_0(0) = 0,
\[ c_0(t) = \frac{-b_2}{2 \cdot k_1 \cdot k_2 \cdot k_3} \cdot \left( (G - F) \cdot (-k_3 - 0.5 \cdot (F - G)) \right) \]  
(Eq. B1)
\[ \cdot \left( (-G - F) \cdot (-h_2 - 0.5 \cdot (F + G)) \right) \cdot \left( (-k_3 - h_3 - 0.5 \cdot (F + G)) \right) \cdot \exp(\lambda_2 \cdot t) \]  
\[ + \frac{b_3}{2 \cdot k_2 \cdot k_3} \cdot \left( (-G - F) \cdot (-k_2 - 0.5 \cdot (F + G)) \right) \cdot \exp(\lambda_3 \cdot t) \]  
(Eq. B2)
\[ c_0(t) = -b_1 \cdot \exp(\lambda_1 \cdot t) - \frac{b_2}{2 \cdot k_3} \cdot \exp(\lambda_2 \cdot t) \]  
\[ + \frac{b_3}{2 \cdot k_3} \cdot \exp(\lambda_3 \cdot t) \]  
(Eq. B3)
\[ c_0(t) = M + b_1 \cdot \exp(\lambda_1 \cdot t) + b_2 \cdot \exp(\lambda_2 \cdot t) + b_3 \cdot \exp(\lambda_3 \cdot t) \]  
(Eq. B4)

with
\[ \lambda_1 = -k_3, \quad \lambda_2 = 0.5 \cdot (F - G), \quad \lambda_3 = 0.5 \cdot (F + G), \quad \lambda_4 = 0 \]  
(Eq. B5.1–B5.4)
\[ F = -k_1 - k_2 - k_3 \]  
(Eq. B6.1)
\[ G = \sqrt{-4 \cdot k_1 \cdot k_2 + (k_1 + k_2 + k_3)^2} \]  
(Eq. B6.2)
\[ b_1 = \frac{k_1 \cdot k_3 \cdot M}{k_1 \cdot (k_2 - k_3) + k_5 \cdot (k_2 - k_3 + k_4)} \]  
(Eq. B6.3)
Acknowledgments—I thank Dr. Stefan Schuster (Friedrich-Schiller Universität, Jena, Germany) for help with the rapid equilibrium approach and fruitful discussions and Dr. Heinz Sklenar (Max Delbrück Center, Berlin, Germany) for advice in Fortran programming and financial support. I am grateful to Dr. Frederick R. Maxfield (Weill Medical College of Cornell University, New York) who gave me advice and the opportunity to use his fluorescent microscopy instrumentation, reagents and cell culture facility for the experiments described in this study. Dr. Allan Wolloff (Albent Einstein College of Medicine, New York) is acknowledged for kindly providing TexRed-ASOR. The editorial board of *J. Lipid Research* is acknowledged for granting the copyright permission to show data previously published in their journal (70).

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