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Acinar Redistribution and Heterogeneity in Transport of the Organic Cation Rhodamine B in Rat Liver

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We studied a possible acinar heterogeneity in the transport of organic cations, using rhodamine B as model compound. Employing perfusions of isolated rat livers in the ante- and retrograde mode and quantitative fluorescence microscopy, Zones 1 and 3 were shown to be equally efficient in taking up rhodamine B. Ten minutes after injection in an antegrade perfusion, 95% of the dose was localized in the portal half of the acinus. Fifty minutes later, however, the amount of rhodamine B in Zone 1 had been reduced to 23%; 30 and 31% were in Zones 2 and 3, respectively, and the medium concentration was doubled. Thus, unchanged rhodamine B appeared to be transported downstream within the liver, either via the medium or directly from cell to cell, finally resulting in a relatively higher rhodamine B concentration in Zone 3. To obtain additional data, we designed a perfusion setup in which the zones could be studied separately. In both zones, the amount excreted into the medium was about 30 times the amount excreted into bile. Intracellular sequestration of rhodamine B and the rate constant for sinusoidal secretion were higher in Zone 3, while the sinusoidal uptake rates were equal; biliary excretion was higher in Zone 1.

Acinar distribution changed with time because rhodamine B, primarily accumulated in Zone 1, was secreted into the sinusoids and taken up again by downstream cells. The finally higher rhodamine B concentration in Zone 3 was caused by a zonal heterogeneity in intracellular sequestration and sinusoidal secretion of rhodamine B.

The hepatic acinus, the microvascular unit of the liver, consists of a heterogeneous hepatocyte population. Sinusoidal blood flows from the terminal portal venule towards the terminal hepatic venule, causing different microenvironments around the periportal (Zone 1) and the perivenous (Zone 3) cells. A heterogeneity is observed not only in structure and metabolic functions (1, 2), but also in transport functions (3–5). In sinusoidal blood, a Zone 1-to-Zone 3 concentration gradient of substances taken up efficiently by the liver was reported (5, 6). Provided that the hepatocytes along the acinus have equal transport characteristics, a similar concentration gradient would be anticipated inside the cells. Jones et al. (7) demonstrated such a gradient for the bile acid derivative, cholylglycylhistamine. Our previous study (8) indicated the gradient for taurocholate to be due to the location of the cells regarding incoming blood, rather than to an intrinsic difference in uptake rate between the zones.

Most of the studies concerning transport heterogeneity deal with organic anions or neutral compounds (9, 10); relatively little is known about the heterogeneity in cation transport. In the present study, we examined a possible intrinsic heterogeneity in the processing of the organic cation rhodamine B (RB) by the liver. Gumucio et al. (11) observed a steep gradient for both RB and acridine orange after 15 min of infusion of a low dose. With a higher dose, the RB gradient disappeared, in contrast to that of acridine orange. To obtain information on the mechanisms underlying the observed heterogeneity in localization of RB, we used the isolated perfused rat liver technique in ante- and retrograde modes, combined with quantitative fluorescence microscopy. Two major observations were made. First, a difference between Zones 1 and 3 in intracellular sequestration and in secretion from the hepatocytes into the sinusoids was demonstrated. Biliary excretion, a saturable process, appeared to be slightly faster from Zone 1. In sinusoidal uptake rate, no heterogeneity existed. Second, following hepatic uptake, an acinar redistribution of RB was seen. This was due to sinusoidal secretion of RB from Zone 1 cells and reuptake in downstream cells. Time-dependent acinar redistribution was not reported before; it may have implications for the interpretation of results obtained from experiments in which selective labeling of one zone is intended.

MATERIALS AND METHODS

Animals. Male Wistar rats were kept in a controlled environment with a 12-hr dark-light cycle. They were fasted overnight before their livers were excised. Body weights averaged 296 ± 6 and 351 ± 48 gm in the first and second protocol perfusions, respectively. The liver-to-body-weight ratio was 3.6 ± 0.5 and 3.3 ± 0.3%, respectively (mean ± S.D.).

Chemicals. Demineralized bovine serum albumin was obtained from Organon Teknika (Oss, The Netherlands), and sodium taurocholate was obtained from Fluka AG (Buchs, SG, Switzerland). RB was obtained from Sigma Chemical Company (St. Louis, Mo.), β-glucuronidase was obtained from Boehringer (Mannheim, Federal Republic of Germany), and thin-layer chromatography plates (G 1500/LS 254) were obtained from Schleicher & Schüll (Dassel, Federal Republic of Germany). All other chemicals were of analytical grade.

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Isolated Perfused Rat Liver. The surgical techniques and design of the perfusion apparatus used in these studies have been described in detail previously (8, 12). Two perfusion protocols were used.

The first protocol was designed to assess the hepatic uptake rate and acinar distribution of RB when administered initially to Zone 1 or 3. One hundred milliliters of Krebs-Henseleit-bicarbonate buffer, containing 0.2% (w/v) glucose and 1% (w/v) bovine serum albumin, were used as perfusion medium. The flow rate was established at 3.5 ml per min per gm liver at a constant portal pressure of 12 cm H_2O. The medium was oxygenated with an oxygen-carbon dioxide mixture (95:5). Throughout each experiment, the pH of the perfusate was monitored and maintained between 7.35 and 7.45, either by slight adjustments in the pCO_2 or by the addition of small amounts of a concentrated sodium bicarbonate solution. The medium recirculated in either the ante- or retrograde direction. The liver was allowed to stabilize for 30 min, during which time an infusion of 15 μmoles per hr taurocholate was started to replace excreted bile salts. At the end of the stabilization period, 120 nmoles RB were injected into the medium reservoir. Bile and perfusate samples were then collected for 120 min at various time intervals. Bile volume was determined gravimetrically, assuming a density of 1 gm per ml.

The second protocol was designed to study the rates of RB transport in Zones 1 and 3 separately. Figure 1 schematically shows the modified perfusion procedure. Until 6 min after administering RB to the medium reservoir, the first protocol was followed. In this way, Zone 1 or 3 was labeled selectively with RB in an ante- or retrograde perfusion, respectively (indicated by ⟶). Then the medium was discarded; the flow was restored at 30 to 35 ml per min in the reverse direction (indicated by ⟶▷), with fresh medium by turning taps a to a^1 (Figure 1). This reversed medium flow prevented the RB, secreted from the labeled hepatocytes into the sinusoids, from reaching the unlabeled zone. Thus, redistribution of RB via the medium towards the unlabeled hepatocytes was not possible in this protocol. The medium used in the single-pass perfusion consisted of Krebs-Henseleit-bicarbonate buffer, with 0.2% (w/v) glucose, 0.2% (w/v) bovine serum albumin and 6.5 μM sodium taurocholate. The portal or hepatic venous outflow, respectively, was diverted at the disconnection site in the tubing (b), and collected at various time intervals in 10-sec aliquots. Bile was collected in 5- or 10-min aliquots. During the reversal of the medium flow, the hepatic circulation was interrupted for less than 2 min.

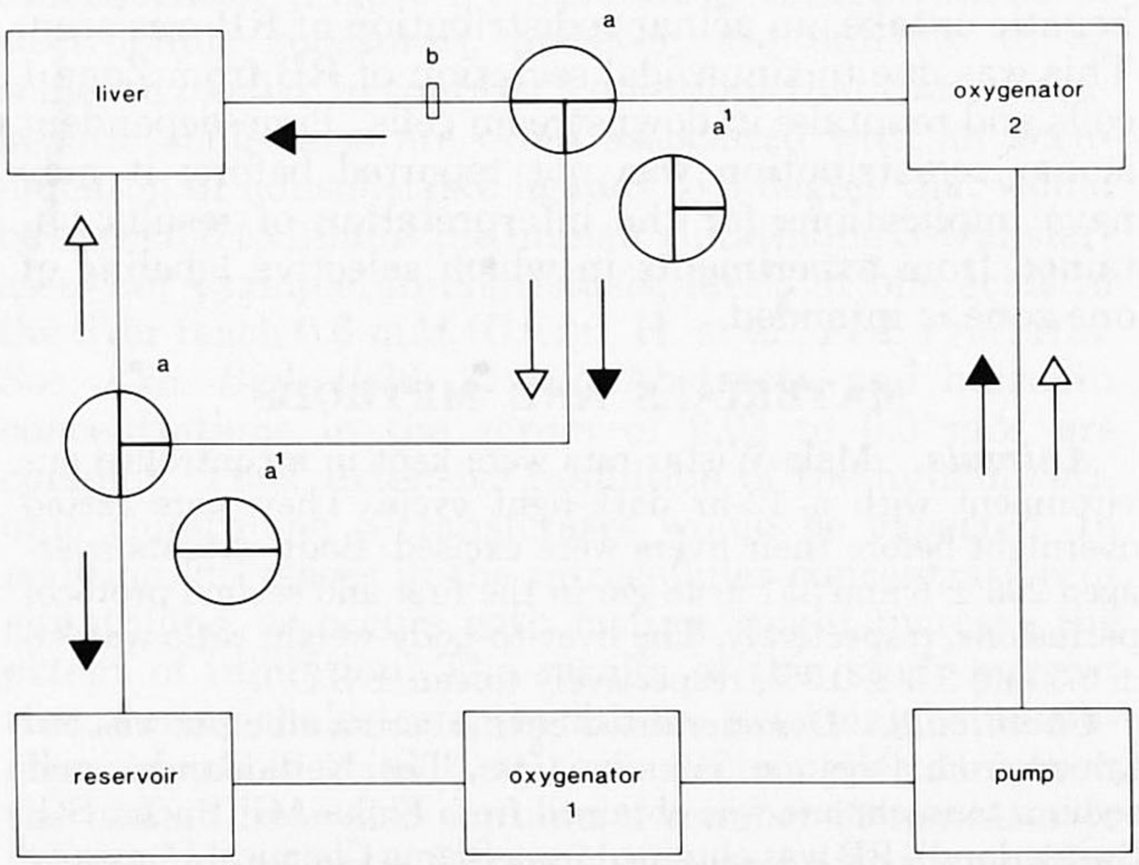

FIG. 1. The isolated perfused rat liver apparatus as used in the second protocol perfusions. The liver was perfused in either the ante- or retrograde recirculating mode for 6 min (⟶). Then, the direction of flow was reversed (⟶▷) by turning taps a to a^1. The medium was collected at the disconnection site b (single-pass).

Fluorescence Microscopy Studies. First protocol perfusions were performed for 10, 20, 30, 40, 50 and 60 min after injecting RB. At the end of each experiment, pieces of liver were excised while perfusion was continued. Within 10 sec after excision, the tissue blocks were snap-frozen in liquid freon (−96°C). In second protocol perfusions, liver pieces were frozen at 20 min after reversion of the flow. Liver sections were cut at 12 μm in a cryostat (−25°C). Of two consecutive sections, the second was stained with hematoxylin and eosin for histological identification. Every first section was allowed to dry on a glass microscope slide and examined unmounted under a Leitz Orthoplan microscope with the appropriate fluorescence filters (excitation = 530 to 560 nm; emission > 580 nm). Photographs were recorded on Kodak Ektachrome II film at 400 ASA. Quantitative fluorescence microphotometry was performed to quantitate the observations. Fluorescence was linearly correlated with RB concentration (11). The length of the acinus was scanned in steps of about 40 μm (about two hepatocytes) on a Leitz MPV2 fluorescence microscope. The fluorescence of the sample was excited by a HBO 100 Hg lamp by epiillumination through the objective of the microscope. An area of about 1,200 μm^2 was measured. About 10 acini were examined at each time point.

Analytical Procedures. At the end of each experiment, the liver was removed and homogenized in water (1:4). The homogenates were extracted with 5 volumes of 80% methanol (4°C). Bile and medium samples were diluted with water, 80 and 10 times, respectively. Fluorescence was measured on an Aminco-Bowman spectrophotofluorimeter (excitation = 540 nm; emission = 580 nm; slits 1 and 10 nm, respectively). The amount of RB present in the liver was calculated using the following mass balances: at any time point during a recirculating perfusion, the sum of the amount of RB in the liver, the amount in the medium, the amount sampled from the medium and the amount excreted into bile until that time equals the injected dose of RB. In this way, we also calculated the amount of RB present in the liver at the beginning of the single-pass perfusion in the second protocol (RB liver load). At any time point during the single-pass perfusion, the sum of the amount present in the liver and the amounts of RB excreted into bile and medium until that time equals this RB liver load.

Glucuronic acid conjugates of RB were identified by incubation with β-glucuronidase. One hundred microliters of a sample of bile, medium or homogenate were mixed with 100 μl 200 mM sodium acetate buffer (pH 6.0) and 20 μl β-glucuronidase. The mixture was incubated for 1, 3 or 24 hr at 37°C. Fluorescence was determined before and after incubation, together with the necessary controls (the mixture without β-glucuronidase or without the buffer).

Thin-Layer Chromatography. Liver extracts and β-glucuronidase incubation mixtures, prepared as described above (20 μl), and bile samples (10 μl) were chromatographed directly. Medium extracts were prepared as follows. Protein was removed from 80 ml medium by pressure dialysis in a Miniconcentrator, fitted with a PM-10 membrane (both from Amicon BV, The Netherlands). The ultrafiltrate was freeze-dried and the residue extracted with 2 ml methanol; 50 μl of this extract was spotted on the thin-layer chromatography plate. Control experiments with bile ascertained that no metabolite or parent compound was lost or decomposed during both extraction procedures. The plate was then placed in a chamber saturated with 80% N-propanol-20% formic acid and developed for 2.5 hr. Fluorescence was observed in the light of a 366 nm Hg lamp.

RESULTS

Hepatic Uptake and Distribution. When administered to the perfusion medium in an antegrade recirculating perfusion (first protocol), RB was removed efficiently by the liver (Figure 2); the initial half-life was 2.0 min. With a medium volume of 100 ml, the initial clearance was 34.7 ml per min (Cl = kV), about the same as the medium flow (35 ml/min). So the initial extraction approached 100%, and in 10 min, 95% of the dose was taken up into the portal half of the acinus (Figure 3A). About 5% of the dose remained in the medium. Twenty minutes after the injection of RB, the fluorescence had spread towards Zone 3; 10 min later, a steady-state was reached in which the fluorescence intensity was even higher in Zone 3 than in Zone 1. Until at least 2 hr after injection, this distribution remained the same. Thus, an intrahepatic redistribution of fluorescence had occurred, leading to a higher RB concentration in Zone 3. Figure 3B shows the fluorescence pattern at 60 min after injection. At this time, the amount of RB in the liver was 84% of the injected dose, the amount in the medium nearly doubled to 9% of the dose and only 7% had been excreted into bile.

The medium and bile curves from a retrograde perfusion were identical to the ones from an antegrade recirculating perfusion as described in Figure 2 (results not shown). Ten minutes after injection in a retrograde perfusion, RB fluorescence was localized in Zone 3 (Figure 3C).

Figure 4 shows the quantified fluorescence levels of liver sections from the same points of time as the sections depicted in Figure 3. The fluorescence curves obtained after 10 min of ante- or retrograde perfusion were each other's mirror image, with half the acinus length as the mirror axis. This means that, under equal circumstances, in Zones 1 and 3 an equal number of cells was labeled with RB.

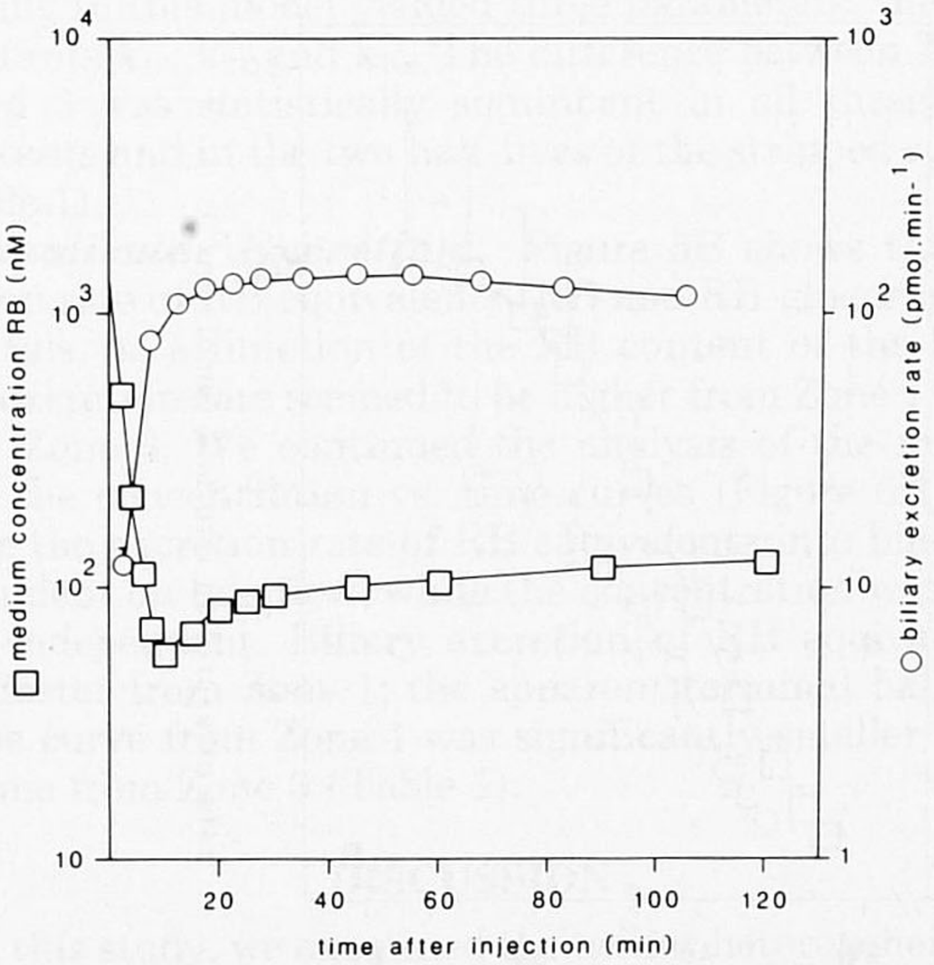

FIG. 2. Medium concentration (□) and biliary excretion rate (○) vs. time curves of 120 nmoles RB injected into the medium of an antegrade recirculating perfusion. Mean of four experiments (S.E. was smaller than symbols).

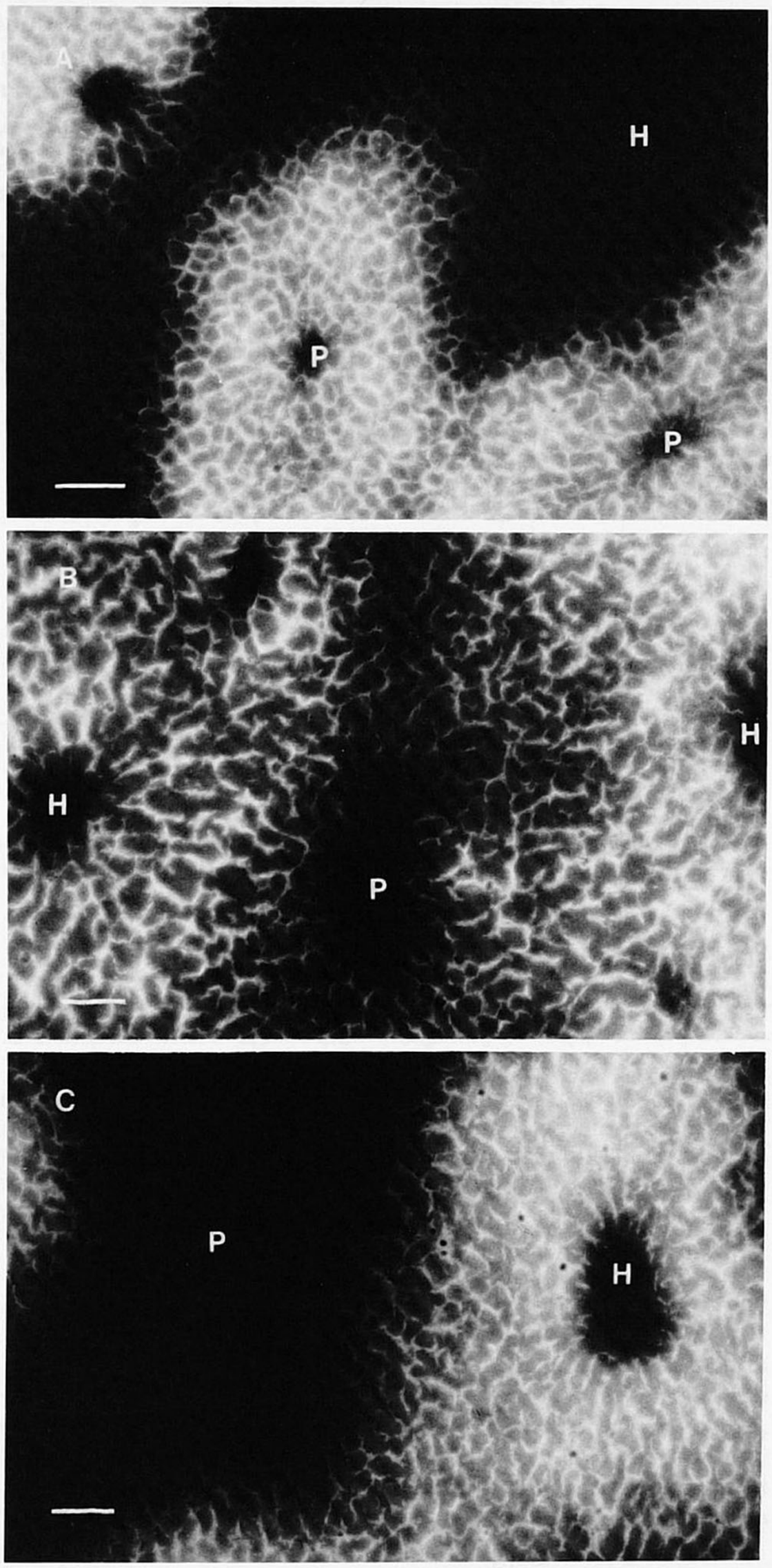

FIG. 3. Fluorescence photomicrographs of RB localization in the liver. Ten (A) and 60 (B) min after injection in an antegrade perfusion, and 10 min after injection in a retrograde perfusion (C). Experimental conditions were as in Figure 2. P = portal venule; H = hepatic venule; *bar* = 25 μm.

The area under the fluorescence-distance curves (Figure 4) was taken as a measure of the total amount of RB present in the liver, being 95% of the injected dose at 10 min and 84% at 60 min after injection. The fluorescence measured here originated from intra- and extracellular RB. We neglected extracellular fluorescence because the amount of RB in intrahepatic bile and plasma was very low. Intrahepatic bile [about 4.3 μl per gm liver (13)] contained maximally 0.4% of the injected dose, and in-

trahepatic plasma [the extracellular volume was about 1.6 ml (13)] contained a maximum of 0.15% of the dose. We divided the acinus arbitrarily into three equally large zones to calculate the amount of RB in each zone: 10 min after injection, 69% of the injected dose was localized in Zone 1, 24% in Zone 2 and 2% in Zone 3. Fifty minutes later, the amounts were 23, 30 and 31%, respectively.

Metabolism. In perfusion medium and in the liver, no metabolites could be detected with the chromatographic method used. Yet, RB was excreted into bile predominantly as a glucuronide, as shown by the β-glucuronidase treatment. Fluorescence characteristics (intensity and peak wavelengths) were the same for RB and RB-glucuronide. Next to this conjugate, very little unchanged RB and some other metabolites—probably the *N*-deethylated derivatives of RB (14)—were excreted into bile, altogether being less than 2% of the dose.

Sinusoidal Secretion. To study the transport characteristics of Zones 1 and 3 separately, it was necessary to label each zone selectively with RB. In a recirculating perfusion, this was achieved in 10 min; but 20 min later, RB was redistributed over the acinus. To keep the labeling restricted to one zone throughout the experiment, we designed the second perfusion protocol. In this protocol, only one zone was labeled during a recirculating perfusion, lasting at most 10 min; Zone 1, for instance, was labeled in an antegrade perfusion. By reversing the medium flow and starting a single-pass perfusion with new medium, RB secreted from Zone 1 into the sinusoids could no longer reach Zone 3. In this way, fluorescence measured in bile and medium originated from Zone 1 alone. The same experiment was performed with Zone 3 by loading this zone in a retrograde perfusion.

In 90 min, 2.8 ± 1.0% of the RB liver load was excreted into bile and 76.0 ± 3.0% into medium from Zone 1; from Zone 3, the amounts were 2.2 ± 0.7 and 67.7 ± 1.9%, respectively (mean ± S.D.; n = 4). Acinar redistribution was virtually absent, as shown by fluorescence microscopy. This implies that, in first protocol perfusions, RB was redistributed mainly via the medium and not by direct cell-to-cell transport.

At various points of time, the amount of RB in the liver was calculated as described in "Materials and Methods." Figure 5A shows the excretion rate into medium as a function of this amount, which is present only in Zone 1 or Zone 3. At a RB content of 65 nmoles or higher, the excretion rate was higher from Zone 3 than from Zone 1. Below 65 nmoles, this relation was reversed.

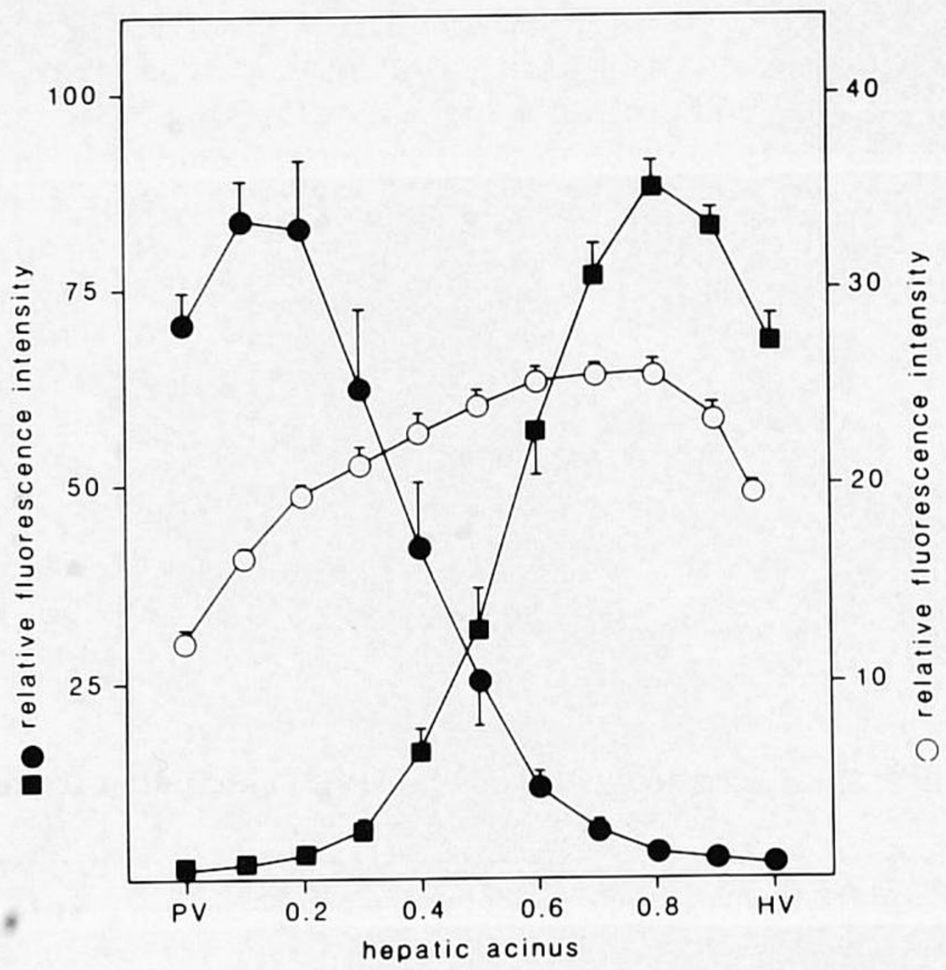

FIG. 4. Quantitative determination of RB fluorescence across the acinus. Experimental conditions were as in Figures 2 and 3. ■ = 10 min after injection, retrograde perfusion; ● = 10 min after injection, antegrade perfusion; ○ = 60 min after injection, antegrade perfusion; PV = portal venule; HV = hepatic venule; mean ± SE; n = 8 to 10 acini.

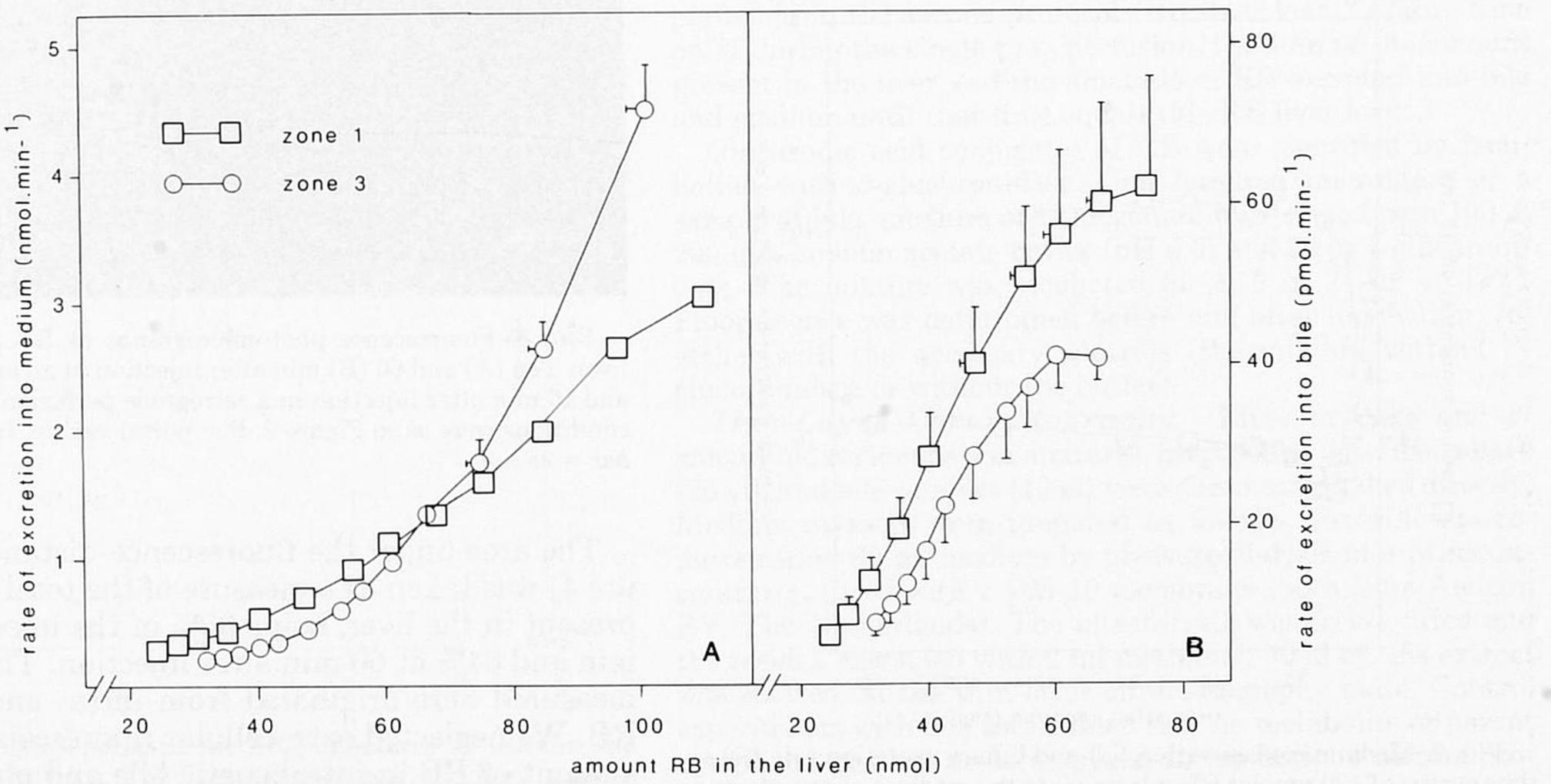

FIG. 5. Excretion rates into medium (A) and bile (B) as a function of the RB content of the liver in the nonrecirculating period of the second protocol perfusion, after selectively loading Zone 1 or 3. Mean ± SE; n = 4 (S.E. often smaller than symbols).

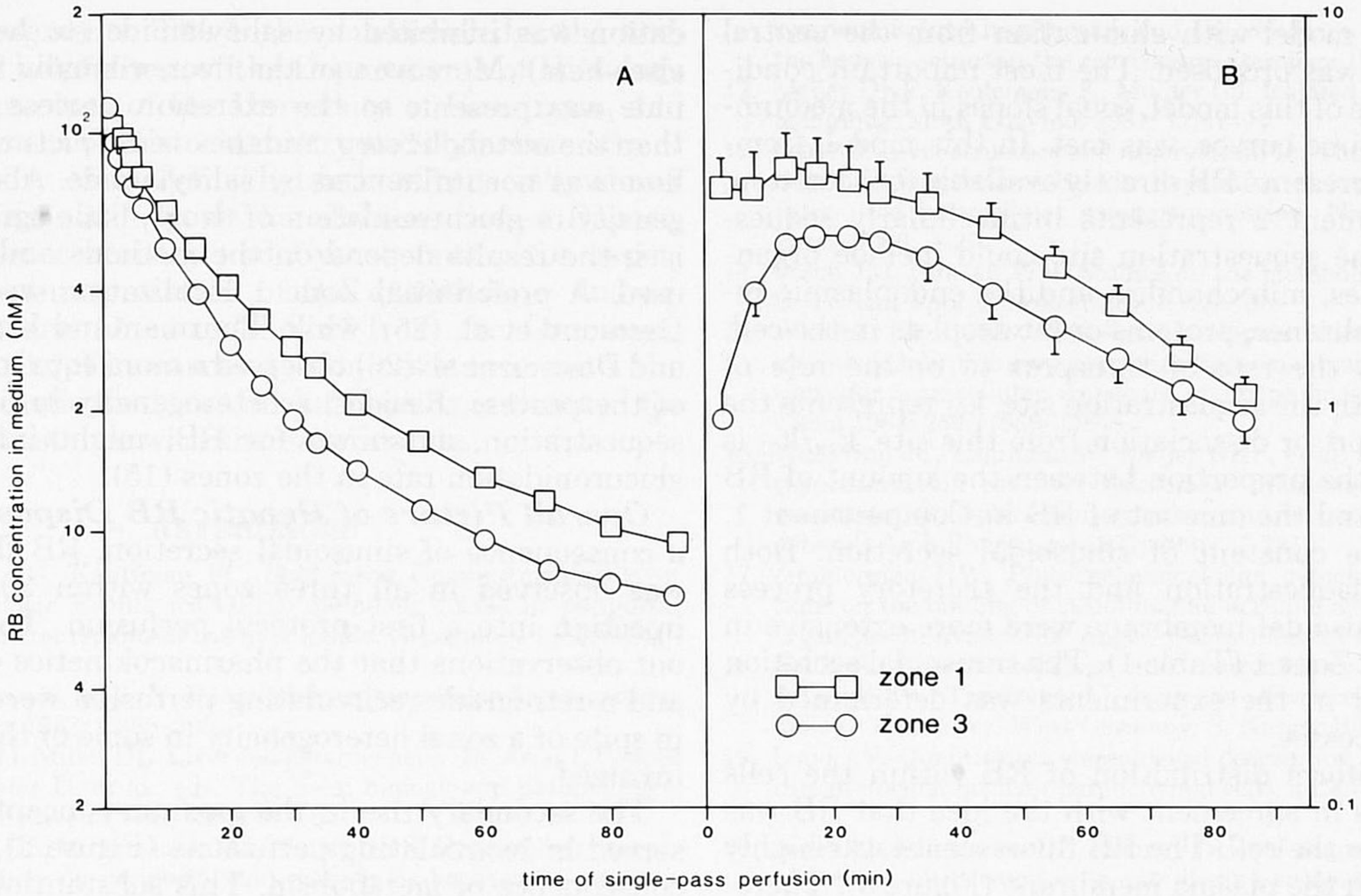

FIG. 6. Concentration of RB equivalents in medium (A) and bile (B) vs. time in the nonrecirculating period of the second protocol perfusion, after selectively loading Zone 1 or 3. Mean ± SE; n = 4 (S.E. often smaller than symbols).

The results of these experiments were further analyzed by using the medium concentration vs. time graphs of Zones 1 and 3 (Figure 6A). The graphs were biphasic, and their slopes were similar to the corresponding slopes of the RB content of the liver vs. time graphs (curves not shown). They could be analyzed according to a two-compartment model, with elimination from the central compartment. In these calculations, biliary excretion was neglected because the amount excreted via bile was very low compared to the sinusoidal secretion. Analysis according to this model yielded three parameters, the rate constants k_{12}, k_{21} and k_{10}. The difference between Zones 1 and 3 was statistically significant in all three rate constants and in the two half-lives of the stripped curves (Table 1).

Canalicular Excretion. Figure 5B shows the excretion rate of RB equivalents (RB and RB-glucuronide) into bile, as a function of the RB content of the liver. The excretion rate seemed to be higher from Zone 1 than from Zone 3. We continued the analysis of the results with the concentration vs. time curves (Figure 6B) because the excretion rate of RB equivalents into bile was dependent on bile flow, while the concentration was bile flow-independent. Biliary excretion of RB equivalents was faster from Zone 1; the apparent terminal half-life of the curve from Zone 1 was significantly smaller than the one from Zone 3 (Table 1).

TABLE 1. Pharmacokinetic parameters calculated from the second protocol perfusions

	Zone 1	Zone 3	p[a]
Medium			
$t_{1/2}$ (1)	9.5 ± 0.6	7.7 ± 0.5	<0.05
$t_{1/2}$ (2)	50.5 ± 4.2	77.5 ± 4.7	<0.005
k_{12}	0.023 ± 0.002	0.037 ± 0.005	<0.025
k_{21}	0.032 ± 0.003	0.018 ± 0.001	<0.005
k_{10}	0.033 ± 0.002	0.045 ± 0.003	<0.01
k_{12}/k_{21}	0.76 ± 0.07	2.01 ± 0.18	<0.0005
Bile			
$t_{1/2}$ (concentration)	25.9 ± 0.7	36.0 ± 2.3	<0.001

The half-lives of the first [$t_{1/2}$ (1)] and second [$t_{1/2}$ (2)] phases were calculated (in minutes) from the medium concentration vs. time curves. Rate constants (k_{12}, k_{21} and k_{10}) were calculated (in minutes^{-1}) according to a two-compartment model with elimination from Compartment 1. In addition, the apparent terminal half-lives of the bile concentration vs. time curves are given. Mean ± SE (n = 4).

[a] Student's t test was used to determine statistical significance.

DISCUSSION

In this study, we examined the acinar heterogeneity of the major processes involved in hepatic disposition of RB: sinusoidal uptake; sinusoidal secretion, and canalicular excretion.

Hepatic Uptake. As indicated previously (10), we cannot conclude from Figure 2 whether the sinusoidal uptake of RB was heterogeneous. Figure 4, however, showed that in Zones 1 and 3, an equal number of cells was involved in the uptake of an equal amount of RB. This implies that the rates of uptake were the same in both zones. Therefore, a heterogeneity in hepatic uptake of RB was rebutted.

Sinusoidal Secretion. This process was studied in the second protocol perfusions. Sinusoidal secretion appeared to be substantial, exceeding the biliary excretion 30 times.

To analyze the concentration vs. time curves, a two-

compartment model with elimination from the central compartment was proposed. The most important condition for the use of this model, equal slopes in the medium- and liver vs. time curves, was met. In this model, Compartment 1 represents RB directly available for secretion, and Compartment 2 represents intracellularly sequestrated RB. The sequestration site could include organelles (lysosomes, mitochondria and the endoplasmic reticulum), membranes, proteins or fat droplets in the cell. k_{12} represents the rate of transport to or the rate of association with the sequestration site. k_{21} represents the rate of transport or dissociation from this site. k_{12}/k_{21} is a measure of the proportion between the amount of RB sequestrated and the amount of RB in Compartment 1. k_{10} is the rate constant of sinusoidal secretion. Both intracellular sequestration and the secretory process across the sinusoidal membrane were more extensive in Zone 3 then in Zone 1 (Table 1). The sinusoidal secretion rate measured in the experiments was determined by these two processes.

The nonuniform distribution of RB within the cells (Figure 3) was in agreement with the idea that RB was sequestrated in the cell. The RB fluorescence was highly associated with the plasma membrane (Figure 3). Therefore, once inside the cell, RB will probably partition between cytosolic binding proteins and *intra*cellular membranes, depending upon the relative magnitude of these pools, and the relative affinities of the membranes and proteins for the substrate. This was also observed for bilirubin, like RB a lipophilic compound (15). Next to the aforementioned binding sites, RB might be accumulated in mitochondria and lysosomes, as was described for other organic cations (16, 17). Despite numerous investigations on differences in hepatocyte ultrastructure in the acinus concerning endoplasmic reticulum (18–20), binding proteins (21–23) and mitochondria and lysosomes (19, 20, 24), the available data are rather controversial. The present observations, therefore, cannot be easily explained within this context.

Besides a larger extent of sequestration, the rate constant of sinusoidal secretion (k_{10}) of RB was larger in Zone 3. The rate constant of uptake, k_{01}, was large in a recirculating perfusion but could be neglected in these experiments. Because the amount of RB in the sinusoids was very low compared to the amount of RB in the liver, the rate of transport from medium to liver ($k_{01} \times$ the amount of RB in the medium) was negligible compared to the rate of transport from liver to medium ($k_{10} \times$ the amount of RB in the liver). Therefore, the contribution of k_{01} to membrane transport could be neglected in the calculations.

Canalicular Excretion. The biliary excretion of RB equivalents was faster from Zone 1 than from Zone 3. The elimination into bile consisted of at least two processes: the glucuronidation of RB and the transport of the conjugate (and some RB and other metabolites) across the canalicular membrane. Both processes could be rate-limiting and saturable. Glucuronidation most likely was the rate-limiting step in excretion, because preliminary experiments indicated that the biliary excretion of RB equivalents diminished when the glucuronidation was inhibited by salicylamide (to be published elsewhere). Moreover, in the liver, virtually no glucuronide was present, so the excretion process was faster than the metabolic step, and the overall picture presented here was not influenced by salicylamide. About heterogeneity in glucuronidation of drugs, little agreement exists; the results depend on the methods and substrates used. A preferential Zone 1 localization was found by Desmond et al. (25), while Thurman and Kauffman (1) and Dawson et al. (26) observed a more equal distribution of the process. Besides, a heterogeneity in intracellular sequestration, as shown for RB, might influence the glucuronidation rate in the zones (15).

Overall Picture of Hepatic RB Disposition. As a consequence of sinusoidal secretion, RB fluorescence was observed in all three zones within 30 min after injection into a first protocol perfusion. This explains our observations that the pharmacokinetics of an ante- and a retrograde recirculating perfusion were the same, in spite of a zonal heterogeneity in some of the processes involved.

The secondary rise in the medium concentration, observed in recirculating perfusions (Figure 2), was not a consequence of metabolism. This substantial sinusoidal secretion of unchanged RB also caused the redistribution towards Zone 3. Direct cell-to-cell transport was very slow, as was observed in the second protocol perfusions. Because the amount of RB equivalents excreted into bile was very low, a pseudoequilibrium was reached in which the respective amounts of RB in the zones were determined by the rate constants of sinusoidal transport and intracellular sequestration. The eventually higher RB concentration in Zone 3 (Figure 3B) can be explained by a higher excretion rate from Zone 1 at an RB liver content below 65 nmoles per zone (Figure 5A). After 30 min of a first protocol perfusion, the amount of RB was about 30 nmoles in each zone.

A discrepancy existed in the two sinusoidal transport processes: uptake was homogeneously distributed, while secretion was heterogeneous. It could be a question of two different transport systems, but the assumption of a single system can also offer an explanation. A rate constant is a parameter that depends on both clearance and volume of distribution. If one transport system is assumed, clearance, representing the transport process, must be the same for both transport directions. The intracellular volume of distribution, however, might be different between the zones, because interzonal differences in intracellular sequestration have been demonstrated. The extracellular volume of distribution, the medium volume, was the same for both zones.

In conclusion, we observed an acinar heterogeneity in hepatic disposition of RB, with Zone 1 dominating in glucuronidation and Zone 3 in sinusoidal secretion, while sinusoidal uptake was equal in Zones 1 and 3.

Whether these results are representative for (lipophilic) organic cations in general or for RB specifically remains to be established. However, a time-dependent acinar redistribution of substrates has not been demonstrated before and should be taken into account in other studies, in which selective zonal labeling is intended.

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