Processing of the phospholipid analogue phosphatidyl(*N*-sulphorhodamine B sulphonyl)ethanolamine by rat hepatocytes *in vitro* and *in vivo*

Henkjan J. VERKADE,*§ Kristien J. M. ZAAL,† Johannes T. P. DERKSEN,†‡ Roel J. VONK,* Dick HOEKSTRA,† Folkert KUIPERS* and Gerrit L. SCHERPHOF†

*Department of Pediatrics and †Laboratory of Physiological Chemistry, University of Groningen, 10 Bloemensingel, 9712 KZ Groningen, The Netherlands

We have investigated the processing of the non-exchangeable fluorescent phospholipid analogue phosphatidyl(*N*-sulphorhodamine B sulphonyl)ethanolamine (*N*-Rh-PE) by rat liver cells. In the hepatocyte couplet system, *N*-Rh-PE was incorporated into the plasma membrane at 2 °C and readily internalized upon warming to 37 °C. Fluorescence was initially found to be concentrated in vesicles clustered throughout the cell, but subsequently it started to accumulate in pericanalicular vesicles, tentatively identified as lysosomes, and in the bile canaliculus lumen. Analysis of cells and media by t.l.c. revealed the slow formation of at least two metabolites. After intravenous injection into bile-fistula rats of [9,10-³H-*oleoyl*]*N*-Rh-PE incorporated in small unilamellar liposomes, the initial rates of elimination from plasma of ³H and rhodamine label were virtually identical. However, biliary secretion of the ³H label (5.5% of dose at 2 h) was much slower than that of the rhodamine label (49.3% at 2 h). The rhodamine label in bile was chloroform-soluble, but not identical to the native molecule, and was resistant to phospholipase A₂ and alkaline hydrolysis. To gain insight in the mechanism of the rapid bile secretion of this metabolite, we compared the processing of *N*-Rh-PE, its deacylated form [glycerophospho(*N*-sulphorhodamine B sulphonyl)ethanolamine; Gly-*N*-Rh] and the rhodamine label itself (sulphorhodamine B sulphonyl chloride; SRho). Intravenous injection of chloroform-soluble *N*-Rh-PE and of methanol/water-soluble Gly-*N*-Rh complexed with albumin both resulted in rapid bile secretion of chloroform-soluble fluorescent compounds (60.2% and 86.3% respectively at 2 h), which showed behaviour identical to that of the metabolite of liposomal *N*-Rh-PE on t.l.c. Methanol/water-soluble SRho was also rapidly secreted into bile (89.5% at 2 h) without being metabolized. Bile secretion of the chloroform-soluble metabolite of *N*-Rh-PE and of SRho was markedly impaired (–31% and –52% respectively) in GY Wistar rats, which express a genetic defect in the hepatobiliary transport of organic anions. Our data show that the rat hepatocyte is capable of modifying the structure of *N*-Rh-PE, a process which proceeds considerably faster *in vivo* than *in vitro*. The chloroform-soluble metabolite is subsequently rapidly removed via the bile. The canaliculus organic anion transporting system, which is deficient in GY rats, appears to be involved in the excretion of this apolar product of hepatic metabolism.

INTRODUCTION

Hepatocytes are polarized cells with well-defined basolateral (sinusoidal) and apical (canalicular) plasma membrane domains of different protein and lipid compositions [1]. Extensive trafficking of lipids occurs within these cells. Endo- and exo-cytotic processes, mainly at the sinusoidal side of the cells, require efficient and well-coordinated membrane fusion events, processes that obviously involve substantial translocation of membrane lipids. In addition, hepatocytes secrete relatively large amounts of cholesterol and phospholipids, as lipoproteins at the sinusoidal side (mainly very-low-density lipoprotein) or as bile lipids at the canalicular pole. It is well established that highly specific selection and sorting processes are involved in the hepatic secretion of phospholipids. Vance and co-workers have shown that specific pools of phospholipids, selected on the basis of their biosynthetic routes, are recruited for assembly of lipoproteins [2–5]. Little is known about the origin of biliary phospholipids (see, e.g., [6–8]). However, the highly specific composition of biliary phospholipids, more than 80% of which consist of phosphatidylcholines

(PCs) with C_{16:0}:C_{18:1} and C_{16:0}:C_{18:2} configurations [8,9], which is distinctly different from that of the canalicular membrane [10], indicates that their secretion is preceded by extensive sorting processes. The mechanisms responsible for the regulation of these selective transport processes are still largely unknown.

Over the past few years, fluorescent lipid analogues have been introduced for the study of lipid transfer and processing in cellular systems *in vitro* (for reviews, see [11–15]). The analogue C₆-7-nitrobenz-2-oxa-1,3-diazole(NBD)-ceramide has recently been employed to monitor intracellular lipid movement in hepatocytes in relation to the process of biliary lipid secretion [16]. In the present study we have used the fluorescent phospholipid derivative phosphatidyl(*N*-sulphorhodamine B sulphonyl)ethanolamine (*N*-Rh-PE) in studies on hepatocytic lipid transfer and processing. *N*-Rh-PE was selected as a potentially interesting analogue, since this headgroup-labelled phospholipid derivative is non-exchangeable, implying that the probe does not move as a monomer through aqueous phases or transfer during aggregation of labelled and non-labelled membranes. Experiments in non-polarized Baby Hamster Kidney cells have provided

Abbreviations used: *N*-Rh-PE, phosphatidyl(*N*-sulphorhodamine B sulphonyl)ethanolamine; Gly-*N*-Rh, glycerophospho(*N*-sulphorhodamine B sulphonyl)ethanolamine; SRho, sulphorhodamine B sulphonyl chloride; PC, phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; SUV, small unilamellar vesicles; GY rats, Groningen Yellow mutant Wistar rats; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

‡ Present address: ATO-AGRO Technology, Post Box 17, 6700 AA Wageningen, The Netherlands.

§ To whom correspondence and reprint requests should be sent.

evidence that *N*-Rh-PE acts as a marker for membrane trafficking during endocytotic processes [17]. In the present study, the hepatic processing of *N*-Rh-PE was studied *in vitro* in rat hepatocyte couplets, i.e. cells that maintain or regain their polarity in culture [18], and *in vivo* in chronically bile-diverted rats [19]. Part of this work has been presented at the Convention of the American Association for the Study of Liver Diseases, New Orleans, May 1988 [20].

MATERIALS AND METHODS

Materials

Egg-yolk PC (type V-E), cholesterol (type CH-S), Hepes, BSA (fraction V) and *Crotalus adamanteus* venom phospholipase A₂ (EC 3.1.1.4) were obtained from Sigma (St. Louis, MO, U.S.A.). Phosphatidylserine (bovine brain) and *N*-Rh-PE were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Glycerophospho(*N*-sulphorhodamine B sulphonyl)ethanolamine (Gly-*N*-Rh) was prepared from *N*-Rh-PE by alkaline hydrolysis (0.2 M-NaOH in methanol, overnight at room temperature). Sulphorhodamine B sulphonyl chloride (SRho) was from Kodak (Rochester, NY, U.S.A.). The molecular structures of the fluorescent compounds are shown in Fig. 1. [¹⁴C-choline]Dipalmitoyl-PC (DPPC) was synthesized as described by Stoffel [21]. [9,10(n)-³H]Oleic acid (2–10 Ci/mmol) and [7(n)-³H]cholesterol (8 Ci/mmol) were from Amersham. L- α -Di-[1-¹⁴C-oleoyl]PC (80–120 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). 2-[³H-oleoyl]*N*-Rh-PE was prepared by partial deacylation of *N*-Rh-PE with *Crotalus adamanteus* venom

phospholipase A₂ and subsequent reacylation with [³H]oleic anhydride as described by Radakrishnan *et al.* [22]. The doubly labelled product gave a single spot upon t.l.c. analysis, with an *R_F* value identical to that of non-radioactive *N*-Rh-PE (see 'Analyses' below).

Rats

Male Wistar rats (body wt. 200–250 g) were used for the isolation of hepatocyte couplets. For the *in vivo* experiments, male Wistar (NW) and mutant GY (Groningen Yellow) Wistar rats of 250–300 g were equipped with permanent silastic catheters in bile duct and heart, as described in detail elsewhere [19]. The animals were allowed to recover from surgery for 1 week, during which time a steady state in bile acid synthesis is established [19]. GY rats express a congenital defect in the hepatobiliary transport of organic anions, resulting in conjugated hyperbilirubinaemia; details of this rat strain can be found in ref. [23].

Liposomes

For the preparation of liposomes, aliquots of stock solutions in chloroform/methanol (4:1, v/v) of egg PC, cholesterol, phosphatidylserine and *N*-Rh-PE were mixed in a molar ratio of 4:5:1:0.05. Tracer amounts of [³H-oleoyl]*N*-Rh-PE and [¹⁴C-choline]DPPC were added to this mixture when required. The solvent was evaporated under a stream of nitrogen; then the lipid film was dissolved in cyclohexane and lyophilized. To prepare small unilamellar vesicles (SUV), 1.0 ml of Hepes (10 mmol/l)/NaCl (135 mmol/l) buffer, pH 7.4, was added to 20 μ mol of dried lipid and vigorously vortexed. The resulting suspension was sonicated for 60 min with a probe-type sonifier (Branson B12; output 40 W). The optically clear suspension was centrifuged for 15 min at 10000 *g* to remove titanium particles and large liposomes.

Preparation of hepatocyte couplets

Hepatocyte couplets were prepared by the non-circulating collagenase perfusion method of Seglen [24], as modified by Graf *et al.* [18] for preparation of couplets, with additional minor modifications made in our laboratory. In short, rats were anaesthetized with sodium pentobarbital and the liver was perfused for 10 min with calcium- and magnesium-free Hanks' Balanced Salt solution (gassed with 95% O₂/5% CO₂ mixture), followed by perfusion with 200 ml of Leibowitz-15 (L-15) medium containing 90 mg of collagenase (Type VI; Biochrom, Berlin, Germany), all at a flow rate of 20 ml/min. The liver was minced in trypsin-inhibitor-containing L-15 medium (Sigma) and filtered over a 100 mesh nylon filter. The cells were washed twice in L-15 medium and collected by gravity. All solutions were kept at 37 °C and set to pH 7.4. The cells were allowed to attach to coated glass coverslips [coated by incubation for 5 min at room temperature in 50 μ l of poly(L-lysine) containing demineralized water; final concentration 100 μ g/ml], and further kept in maintenance culture for 4–10 h in L-15 medium.

Cell labelling and microscopy

N-Rh-PE was inserted into the plasma membrane of the cells at 2 °C by the ethanol injection procedure, as described by Kok *et al.* [17]. Internalization of the analogue was triggered by raising the incubation temperature to 37 °C. The intracellular fate of the analogue was monitored by fluorescence microscopy, using a Leitz Orthoplan microscope.

Animal experiments

Disappearance of rhodamine and radiolabel from plasma and their secretion into bile were determined in unanaesthetized male rats on the 8th day after surgery.

Fig. 1. Molecular structures of fluorescent compounds

The molecular structure of rhodamine B (Rhob) differs from that of SRho by the presence of an H atom instead of sulphonyl chloride, and a carboxylic group instead of a thiol group.

Small unilamellar vesicles (SUV) labelled with [^3H -oleoyl] N -Rh-PE and [^{14}C -choline]DPPC were injected at a dose of 2 μmol of liposomal lipid/100 g body wt. Injected volumes varied between 0.5 and 1.0 ml/rat. Blood samples of 0.2 ml were collected via the heart catheter at 5, 15, 30, 60, 120, 180 and 240 min after injection, and bile was collected continuously in 15 min fractions using a fraction collector.

To study possible dissociation and/or metabolism of liposomal lipids in plasma, SUV were triply labelled with N -Rh-PE, [^3H]cholesterol and di-[1- ^{14}C -oleoyl]PC and incubated *in vitro* in rat plasma (45 min, 37 °C). Liposomes were re-isolated by gel filtration chromatography (Sephacrose CL-4B, using Hepes/NaCl, pH 7.4, as elution buffer). The eluted fractions were analysed for rhodamine, ^3H and ^{14}C content. Furthermore, rhodamine label was analysed by t.l.c., by procedures described in the 'Analyses' section below.

For comparison of bile secretion patterns of chloroform-soluble N -Rh-PE and the methanol/water-soluble compounds Gly- N -RH and SRho, these three compounds were dissolved in 4% albumin in phosphate-buffered saline (10 mM-sodium phosphate pH 7.4). Administered doses were 30 nmol/rat for N -Rh-PE and Gly- N -Rh, and 52 nmol/rat for SRho. Sampling of blood and bile was performed as described for the liposomal lipids.

Analyses

Rhodamine fluorescence was measured in plasma and in bile using a Perkin-Elmer MPF-43 spectrofluorimeter at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. ^3H radioactivity was determined using Plasmasol (Packard) as a scintillation cocktail in an LKB liquid scintillation counter. Bile samples were first decolourized by addition of an equal volume of 30% H_2O_2 .

Lipids were extracted from bile samples as described by Bligh & Dyer [25], and analysed by t.l.c. on Silica F254 h.p.t.l.c. plates (0.25 mm; Merck, Darmstadt, Germany) with various solvent systems: solvent I, petroleum benzine/diethyl ether/formic acid (60:40:1.5, by vol.); solvent II, chloroform/methanol/conc. HCl (87:13:0.5, by vol.); solvent III, chloroform/methanol/water (65:25:4, by vol.); solvent IV, chloroform/methanol/water/ammonia (65:25:4:1, by vol.).

Biliary and liposomal phospholipid concentrations were determined by phosphorus assay after HClO_4 treatment [26].

Calculations and statistics

Biliary output rates of rhodamine label and radioactivity were determined by multiplying bile flow by bile concentration after correction of the latter for the dead space of the extracorporeal tubing systems. Values are presented as means $\pm$ S.E.M. Significance of differences was assessed by Student's t test, at a $P < 0.05$ level of significance.

RESULTS

Transport and processing of N -Rh-PE by hepatocyte couplets

N -Rh-PE was inserted into the plasma membrane of hepatocyte couplets at 2 °C as described in the Materials and methods section (Fig. 2a). After removal of non-incorporated lipid and increasing the temperature to 37 °C, the label was internalized by the cells. After 30 min at 37 °C, the fluorescence had accumulated in vesicles, distributed in clusters throughout the cell (Fig. 2b). After 60 min, most of the label was seen in vesicles clustered in the pericanalicular region (Fig. 2c). At that time, label was also found in the bile canaliculi (arrow in Fig. 2c). Analysis of fluorescent label extracted from cells and media revealed that

after 60 min of incubation 90% of the label was still present as intact N -Rh-PE. However, the appearance of two additional (chloroform-soluble) spots indicated the occurrence of some intracellular metabolism. One of these metabolites was found to have the same t.l.c. properties as the compound recovered in bile after intravenous injection of N -Rh-PE in *in vivo* experiments (see Tables 1 and 2).

Rhodamine-containing compartments in the hepatocytes were tentatively identified as lysosomes by performing co-localization experiments using Lucifer Yellow as a marker (Figs. 3a and 3b).

Hepatic processing of liposomal [^3H -oleoyl] N -Rh-PE *in vivo*

Fig. 4 shows the plasma disappearance curves of the ^3H , ^{14}C and rhodamine labels after injection of SUV labelled with [^3H -oleoyl] N -Rh-PE and [^{14}C -choline]PC. The ^3H and rhodamine labels were cleared substantially faster from plasma than was the ^{14}C label, which served as a marker for liposome clearance. The plasma disappearance of [^{14}C -choline]PC was similar to that

Fig. 2. Internalization and processing of N -Rh-PE by rat hepatocyte couplets

Cells were labelled at 2 °C with N -Rh-PE in an ethanolic solutions. After raising the temperature to 37 °C, the label was internalized from the plasma membrane into the cell. (a) Cells after labelling at 2 °C. Fluorescence is only seen at the plasma membrane. (b) After 30 min at 37 °C, fluorescence is present in clustered vesicles. (c) After 60 min at 37 °C, fluorescence is found in pericanalicular vesicles and in the canalicular lumen (arrow). (d) Phase contrast image of (c). Magnification $\times 4400$.

Fig. 3. Co-localization of N -Rh-PE and Lucifer Yellow

Cells were labelled at 2 °C with N -Rh-PE in an ethanolic solution. On raising the temperature to 37 °C, the medium of the cells was replaced by medium containing Lucifer Yellow (1 mg/ml). (a) Location of Lucifer Yellow; 30 min at 37 °C. (b) Location of N -Rh-PE in the same cells; 30 min at 37 °C. Magnification $\times 4400$.

Fig. 4. Plasma disappearance of phospholipid labels from SUV

Plasma disappearance of label from [^{14}C -choline]PC ($\square$) and [^3H -oleoyl] N -Rh-PE (^3H , $\circ$; rhodamine, $\bullet$) incorporated into SUV was followed after intracardial injection into rats with permanent catheters in heart and bile duct. SUV, consisting of egg PC, cholesterol, phosphatidylserine and N -Rh-PE in a 4:5:1:0.05 molar ratio were administered in a dose of $2\text{ }\mu\text{mol}$ of lipid/100 g body wt. Blood samples were taken at timed intervals after injection (time zero) and radioactivity and fluorescence were determined in plasma. Data are means $\pm$ S.E.M. of three (^3H), nine (rhodamine) or ten (^{14}C) animals.

Fig. 5. Biliary secretion of radioactivity ($\circ$) and rhodamine fluorescence ($\bullet$) after injection of [^3H -oleoyl] N -Rh-PE-labelled SUV

SUV ($2\text{ }\mu\text{mol}$ of lipid/100 g body wt.) were intracardially administered to rats, as described in the legend to Fig. 4. Bile was collected for 4 h after injection of the liposomes (time zero) in 15 min fractions. Data represent means $\pm$ S.E.M. of four (^3H) or ten (rhodamine) animals.

reported for [^3H]inulin, which is used as an aqueous space marker in liposomes of this size and composition [27,28].

The divergence of the disappearance curves for the ^3H and rhodamine labels after prolonged time intervals is probably explained by re-entry of ^3H label into the plasma compartment. *In vitro*, no indications were found for N -Rh-PE metabolism in plasma: incubation of the N -Rh-PE-containing liposomes with rat plasma, followed by gel filtration chromatography, revealed that all of the rhodamine label remained associated with the liposomal fraction (elution in void volume), and co-eluted with the liposomal markers [^3H]cholesterol and di- ^{14}C -oleoyl]PC. Moreover, t.l.c. analysis showed that the fluorescent label was still in the form of N -Rh-PE.

Fig. 5 shows the processing of N -Rh-PE, monitored by the

Table 1. T.l.c. of N -Rh-PE and its metabolite found in bile after injection of liposomal N -Rh-PE

T.l.c. R_F values are given of injected and biliary secreted compounds containing rhodamine. Compounds were administered to rats equipped with permanent catheters in heart and bile duct, as described in the legend to Fig. 4. Composition of the solvents: I, petroleum benzene/diethyl ether/formic acid, 60:40:1.5 (by vol.); II, chloroform/methanol/conc. HCl, 87:13:0.5 (by vol.); III, chloroform/methanol/water, 65:25:4 (by vol.); IV, chloroform/methanol/water/ammonia, 65:25:4:1 (by vol.).

Solvent	R_F	
	N -Rh-PE	Bile metabolite
I	0	0
II	0.14	0.28
III	0.67	0.67
IV	0.83	0.76

Fig. 6. Gel filtration elution profile of rat bile after intracardial injection of N -Rh-PE-labelled SUV

Gel filtration was performed on Sepharose CL-4B pre-equilibrated and eluted with a bile acid-free HEPES/saline buffer. $\bullet$, Bile acids; $\blacktriangle$, phospholipids; $\blacksquare$, cholesterol; $\circ$, rhodamine. Totally excluded volume (V_0), fraction 20; totally included volume (V_i), fraction 40.

appearance of the [^3H]oleoyl and rhodamine labels in the bile. The rhodamine label was secreted very rapidly ($67.3 \pm 2.2\%$ of dose/4 h) when compared with the ^3H label ($7.2 \pm 0.3\%$ of dose/4 h), indicating intrahepatic dissociation of the two labels.

Radiolabelled and fluorescent metabolites of liposomal N -Rh-PE in bile

The majority of biliary ^3H label derived from liposomal [^3H -oleoyl] N -Rh-PE ($> 80\%$) was methanol/water-soluble; this was not further analysed in these studies. Previous studies [29] showed that a considerable proportion (60–80%) of the [^3H]acyl label from liposomal phospholipids can be recovered in bile as bile acids.

In contrast to the ^3H label, the rhodamine label in bile was exclusively recovered in a chloroform-soluble form. The rhodamine-containing fraction thus obtained was insensitive to phospholipase A_2 , whereas the parent compound N -Rh-PE was readily hydrolysed to lyso- N -Rh-PE. In addition, the biliary product of N -Rh-PE could not be degraded by alkaline hydrolysis (0.2 M-NaOH in methanol, overnight at room temperature), indicative of the absence of carboxyl-ester bonds. Finally, its chromatographic behaviour in four different solvent systems (Table 1) also indicated that the bile compound was not identical to N -Rh-PE. The same compound was also found as a major

Fig. 7. Biliary secretion of rhodamine fluorescence in normal (●) and GY (○) Wistar rats

Biliary rhodamine secretion was followed after intracardial administration of *N*-Rh-PE (a, 30 nmol/rat), SRho (b, 52 nmol/rat) or Gly-*N*-Rh (c, control rats only, 30 nmol/rat) dissolved in 4% albumin. Bile was collected for 2 h after injection, in 15 min fractions. Mean values $\pm$ S.E.M., $n = 3-4$ animals per experiment.

metabolite in the hepatocyte couplet cultures. The bile secretion of the rhodamine label was not affected by a concomitant intraduodenal infusion of taurocholic acid (24 μ mol/h for 5 h; results not shown).

The characteristics of the biliary product derived from liposomal *N*-Rh-PE was further characterized by gel filtration chromatography of native bile, collected during the 2 h after

Table 2. Solubility characteristics and t.l.c. chromatography of *N*-Rh-PE, Gly-*N*-Rh and SRho, and their metabolites found in bile

Shown are solubility characteristics in Bligh & Dyer (B/D) extraction (Chl, chloroform; M/W, methanol/water) and R_F values upon t.l.c. in four different solvent systems of the three injected (IN) rhodamine-containing compounds and their metabolites found in bile (BP, bile products). Composition of the solvent systems can be found in the legend of Table 1.

	<i>N</i> -Rh-PE		Gly- <i>N</i> -Rh		SRho	
	IN	BP	IN	BP	IN	BP
B/D solubility	Chl	Chl	M/W	Chl	M/W	M/W
R_F values						
Solvent I	0	0	0	0	0	0
Solvent II	0.14	0.28	0.06	0.28	0.07	0.07
Solvent III	0.67	0.67	0.32	0.67	0.43	0.43
Solvent IV	0.83	0.76	0.38	0.76	0.51	0.51

injection of *N*-Rh-PE-containing liposomes (Fig. 6). The procedure used, i.e. elution with a bile acid-free buffer, resulted in the separation of a biliary vesicular lipid fraction (cholesterol and phospholipid) and a bile acid fraction, consistent with our previous work [30] and that of others [31–33]. The rhodamine label was eluted near the total volume of the column, distinct from both the vesicular (cholesterol, phospholipids) fractions and the bile acid peak.

Mechanisms of bile secretion of rhodamine-labelled compounds

To gain insight into the mechanisms responsible for the rapid bile secretion of the apolar product of *N*-Rh-PE, we have compared the secretion patterns of fluorescent compounds appearing in bile after injection of *N*-Rh-PE, Gly-*N*-Rh and SRho (for structures, see Fig. 1). Because the latter methanol/water-soluble compounds cannot be incorporated into liposomes, all compounds were injected dissolved in a 4% albumin solution. Furthermore, to investigate whether bile canalicular organic anion transporting systems are involved, we compared biliary secretion in control Wistar rats and mutant GY rats. The three rhodamine-labelled compounds were cleared very rapidly from plasma in both rat strains; fluorescence in plasma was below detectable limits within 15 min after injection (results not shown). Rhodamine label from *N*-Rh-PE was rapidly secreted into bile of control rats, but secretion was markedly delayed in GY rats ($60.2 \pm 1.7\%$ versus $41.4 \pm 2.0\%$ of the administered dose after 2 h respectively, Fig. 7a). Injection of SRho was followed by very efficient secretion in control rats ($89.5 \pm 1.0\%/2$ h), but this was impaired in GY rats ($43.1 \pm 1.2\%/2$ h) (Fig. 7b). The water/methanol-soluble product of saponification of *N*-Rh-PE, i.e. Gly-*N*-Rh, was injected only in control rats (Fig. 7c); rapid bile secretion of rhodamine fluorescence occurred ($86.3 \pm 2.8\%/2$ h).

The fluorescent compounds present in bile were compared by determination of their solubility in Bligh & Dyer [25] extraction phases and their R_F values in various t.l.c. systems (Table 2). No qualitative differences were observed between control and GY rats. The fluorescence found in bile after injection of *N*-Rh-PE was chloroform-soluble and showed identical R_F values upon t.l.c. separation to that of liposomal *N*-Rh-PE (see Table 1). Surprisingly, methanol/water-soluble Gly-*N*-Rh was converted into the same chloroform-soluble metabolite prior to bile secretion. In contrast, SRho appeared to be secreted in an unchanged form.

The apparent involvement of organic anion transporting systems in bile secretion of rhodamine-labelled compounds was

further investigated by measuring the hepatobiliary transport of rhodamine B. This compound is structurally very similar to SRho, but is non-anionic. After intravenous administration of chloroform-soluble rhodamine B to control rats, only $1.42 \pm 0.31\%$ of the dose was secreted into bile within 2 h.

DISCUSSION

Intracellular transport of *N*-Rh-PE in hepatocytes

To explore the possibility of using a non-exchangeable fluorescent phospholipid analogue in studies on intracellular lipid transport processes in hepatocytes, we have investigated the hepatic processing of *N*-Rh-PE both *in vitro* and *in vivo*. The results show that the lipid analogue is processed and partly secreted into bile by hepatocytes, as reflected by accumulation of fluorescence in the canalicular lumen in the couplet system and biliary recovery of fluorescence in bile-diverted rats. However, intact *N*-Rh-PE was never recovered from bile *in vivo*: fluorescence in bile was exclusively present as a chloroform-soluble metabolite. Although present in much smaller amounts, the same metabolite was detected in the couplet system. This metabolite was not formed during incubation of *N*-Rh-PE in rat plasma. These observations suggest that the extensive metabolism of *N*-Rh-PE observed *in vivo* can be attributed to processing of the lipid by hepatocytes.

Initial processing of the lipid probe by hepatocytes *in vitro* probably differed from that *in vivo*, as in the former case the probe was inserted into the plasma membrane of the cultured cells, whereas *in vivo* the major portion of liposomal *N*-Rh-PE is likely to accumulate in the liver by endocytosis of the liposomes. The mode by which albumin-coupled *N*-Rh-PE enters the liver cell *in vivo* is unclear at the moment (see below). However, in the hepatocyte couplets, transport from the sinusoidal membrane to the canalicular lumen appeared to occur predominantly through a vesicular pathway, as the fluorescence, prior to secretion, was concentrated in pericanalicular spots. We tentatively identified these compartments as lysosomes by carrying out co-localization experiments using Lucifer Yellow, analogous to similar experiments with Baby Hamster Kidney cells [17]. In the latter work, evidence was presented that *N*-Rh-PE can be used as a marker for membrane trafficking during endocytosis. By inference, therefore, we conclude that *N*-Rh-PE is processed via lysosomes before its secretion into the bile canalicular lumen, not only *in vitro* but also *in vivo*; it is then secreted into the bile.

A major metabolite in the cell system was presumably identical to the major metabolite formed *in vivo*. The rate at which this product was formed *in vitro* was lower than that observed *in vivo*, which can be readily explained by differences in experimental approach. Appreciable amounts of the metabolite were only detected *in vitro* after incubation times exceeded 60 min. This timing coincides with microscopic observations of vesicle clustering in the pericanalicular region and the appearance of fluorescence in the canalicular lumen. Therefore it is tempting to suggest a direct correlation between lysosomal processing of *N*-Rh-PE and subsequent secretion of fluorescence into bile.

Clearance of *N*-Rh-PE *in vivo*

The liposomes used for studies *in vivo* were of a size (SUV) and a composition known to be taken up rapidly by the liver, predominantly by hepatocytes, where they end up in the lysosomal compartment [28,34]. Surprisingly, we found a preferential plasma disappearance of ^3H -oleoyl and rhodamine labels contained in *N*-Rh-PE compared with other liposomal aqueous phase and membrane markers [27,28,29]. Incubation of liposomes in plasma *in vitro* did not lead to dissociation of *N*-Rh-PE from

the liposomes or to the formation of metabolites of *N*-Rh-PE. Therefore we interpret the plasma disappearance of *N*-Rh-PE acyl and headgroup label as preferential removal of intact *N*-Rh-PE from the liposomes. The mechanism of the accelerated plasma disappearance of [^3H -oleoyl]*N*-Rh-PE is unclear, but the fact that the major fraction of the rhodamine label was secreted rapidly into bile indicates a direct and active involvement of the liver in this uptake process. Crawford *et al.* [16] found an even more striking preferential uptake of C_6 -NBD-ceramide from dioleoyl-PC liposomes in the perfused rat liver system, which they ascribed to transfer of the fluorescent lipid from the liposomes to the plasma membrane. However, as *N*-Rh-PE is a non-exchangeable probe [17], such a mechanism is unlikely in case of this lipid. Plasma disappearance of albumin-associated *N*-Rh-PE, Gly-*N*-Rh and SRho, as well as that of rhodamine B, was very rapid. For the lipophilic compound rhodamine B a high hepatic extraction has been reported previously [35]. Passive uptake appears to constitute the major part of uptake for this particular compound, while carrier-mediated uptake may contribute to a small extent [36]. No data are available on the mechanisms by which the other anionic compounds are eliminated from plasma, but passive uptake by diffusion is unlikely for hydrophilic compounds such as Gly-*N*-Rh and SRho. The rapid appearance of fluorescence in the bile indicates that the liver plays a major role in this process, which may be (partly) mediated by organic anion transporting systems in the sinusoidal membrane.

Bile secretion of *N*-Rh-PE label

Although the rhodamine and ^3H labels of liposomal *N*-Rh-PE were eliminated at similar rates from the plasma, their biliary secretion rates differed greatly, indicating intrahepatic dissociation of the two labels, i.e. extensive metabolism of the phospholipid analogue. Furthermore, as the ^3H label secreted into bile was no longer associated with a chloroform-soluble fraction, substantial catabolism of the parent oleoyl chain must have taken place. Indeed, in a recent study [29] we have shown that PC-derived [^3H]acyl label is recovered in bile mainly as bile acid. In that study we also demonstrated that the ^{14}C -labelled headgroup of PC is secreted into bile as part of a phospholipid molecule and that its secretion can be stimulated by bile acid infusions. The rhodamine label from *N*-Rh-PE, however, was not associated with biliary phospholipids and its secretion could not be stimulated by taurocholic acid administration. This is in contrast with the reported stimulatory effect of taurocholic acid on the biliary secretion of NBD-labelled sphingomyelin and glucosylceramide after loading of the perfused rat liver with C_6 -NBD-ceramide [16].

The nature of the *N*-Rh-PE-derived compound in bile has not yet been identified. However, a complete de-acylation of the phospholipid analogue seems an obligatory step for further hepatic metabolism and efficient bile secretion. The observation that Gly-*N*-Rh gave rise to the same bile product as did *N*-Rh-PE was unexpected, as it implies that a water/methanol-soluble compound is converted into a chloroform-soluble one before secretion into bile. In general, efficient bile secretion of apolar compounds is achieved by conjugation reactions which lead to the formation of anionic, water-soluble metabolites. This observation also indicates that complete de-acylation of *N*-Rh-PE occurs before further metabolism and bile secretion takes place. Furthermore, the observation that SRho was secreted into bile unchanged indicates that the formation of the metabolite of *N*-Rh-PE and Gly-*N*-Rh requires the retention of the amide linkage at the thiol group in the latter molecules. The refractory nature of the bile compound to alkaline hydrolysis excludes (re-)introduction of carboxy esters. Despite its chloroform-solubility,

this metabolite showed no affinity to biliary phospholipid/cholesterol vesicles in gel filtration experiments. These characteristics of the biliary rhodamine compound show that it does not act as a (phospho)lipid marker in bile.

This view is supported by the fact that biliary secretion of the bile product of *N*-Rh-PE, as well as that of SRho, was impaired in GY rats, suggesting involvement of the canalicular organic anion transporting system in the secretion process. Based on these results, it is likely that the presence of the bulky rhodamine label with an anionic thiol group dictates the handling of the *N*-Rh-PE metabolite by the liver cells. The apparent involvement of the organic anion transporting system in the secretion of this chloroform-soluble compound further emphasizes the broad substrate specificity of this secretory system.

It is obvious that, because of the artificial nature and extensive metabolism of *N*-Rh-PE, no conclusions can be drawn as to the physiological significance of the present observations in relation to biliary lipid secretion. Given its behaviour *in vitro* described in this paper, in combination with earlier studies in other cell systems (e.g. [17]), the lipid analogue may well serve as a valuable tool for exploring lipid trafficking pathways related to endocytosis in hepatocytes. The results *in vivo* emphasize that metabolism of *N*-Rh-PE should be taken into account, especially as its metabolite(s) share some characteristics with the original compound, such as chloroform solubility and behaviour on t.l.c. in alkaline systems.

H.J.V. was sponsored by the Academic Hospital Groningen, Stimulation Grant no. SG 23. J.T.P.D. was sponsored by the Netherlands Organization for Scientific Research (Grant no. 900-523-060). F.K. is a Research Fellow of the Royal Netherlands Academy of Arts and Sciences. We thank Rick Havinga and Albert Gerding for analytical support and Lodewijk Martijn for drawing the figures. The technical advice of Professor G. Kurz (Institute for Organic Chemistry and Biochemistry, University of Freiburg, Freiburg, Germany) is gratefully acknowledged.

REFERENCES

- Meier, P. J., Sztul, E. S., Reuben, A. & Boyer, J. L. (1984) *J. Cell Biol.* **98**, 991–1000
- Vance, J. E. & Vance, D. E. (1986) *FEBS Lett.* **204**, 243–246
- Vance, J. E. & Vance, D. E. (1986) *J. Biol. Chem.* **261**, 4486–4491
- Vance, J. E. (1988) *Biochim. Biophys. Acta* **963**, 70–81
- Vance, J. E. (1989) *Biochim. Biophys. Acta* **1006**, 59–69
- Hardison, W. G. M. & Evans, W. H. (1986) *J. Hepatol* **3**, 318–327
- Robins, S. J. & Brunengraber, H. (1982) *J. Lipid Res.* **23**, 604–608
- Coleman, R. (1987) *Biochem. J.* **244**, 249–261
- Balint, J., Kyriakides, E. C., Spitzer, H. L. & Morrison, E. C. (1965) *J. Lipid Res.* **6**, 96–99
- Kawamoto, T., Okano, G. & Akino, T. (1980) *Biochim. Biophys. Acta* **619**, 20–34
- Pagano, R. E. & Sleight, R. G. (1985) *Science* **229**, 1051–1057
- Pagano, R. E. (1990) *Curr. Opin. Cell Biol.* **2**, 652–663
- Hoekstra, D., Eskelinen, S. & Kok, J. W. (1989) in *Organelles in Eukaryotic Cells* (Tager, J. M., Azzi, A., Paapa, S. & Guerrieri, F., eds.), pp. 59–83, Plenum Press, New York
- Dawidowicz, E. A. (1987) *Annu. Rev. Biochem.* **56**, 43–61
- Sleight, R. G. (1987) *Annu. Rev. Physiol.* **49**, 193–208
- Crawford, J. M., Vinter, D. W. & Gollan, J. L. (1991) *Am. J. Physiol.* **260**, G119–G132
- Kok, J. W., Ter Beest, M., Scherphof, G. & Hoekstra, D. (1990) *Eur. J. Cell Biol.* **53**, 173–184
- Graf, J., Gautam, A. & Boyer, J. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6516–6520
- Kuipers, F., Havinga, R., Bosschieter, H., Toorop, G. P., Hindriks, F. R. & Vonk, R. J. (1985) *Gastroenterology* **88**, 403–411
- Kuipers, F., Vonk, R. J., Scherphof, G. L. & Derksen, J. T. P. (1988) *Gastroenterology* **94**, A558
- Stoffel, W. (1975) *Methods Enzymol.* **35**, 533–541
- Radakrishnan, R., Robson, R. J., Takagaki, Y. & Khorana, H. G. (1981) *Methods Enzymol.* **72**, 408–433
- Kuipers, F., Enserink, M., Havinga, R., van der Steen, A. B. M., Hardonk, M. J., Fevery, J. & Vonk, R. J. (1988) *J. Clin. Invest.* **81**, 1593–1599
- Seglen, P. O. (1976) *Methods Cell. Biol.* **13**, 29–83
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Biophys.* **37**, 911–917
- Böttcher, C. J. F., Van Gent, C. M. & Pries, C. (1961) *Anal. Chim. Acta* **24**, 203–204
- Spanjer, H. H. & Scherphof, G. L. (1983) *Biochim. Biophys. Acta* **734**, 40–47
- Spanjer, H. H., Van Galen, M., Roerdink, F., Regts, J. & Scherphof, G. L. (1986) *Biochim. Biophys. Acta* **863**, 224–230
- Verkade, H. J., Derksen, J. T. P., Gerding, A., Scherphof, G. L., Vonk, R. J. & Kuipers, F. (1991) *Biochem. J.* **275**, 139–144
- Verkade, H. J., Wolbers, M. J., Havinga, R., Uges, D. R. A., Vonk, R. J. & Kuipers, F. (1990) *Gastroenterology* **99**, 1485–1492
- Sömjen, G. J. & Gilat, T. (1985) *J. Lipid Res.* **26**, 699–704
- Tazuma, S. & Holzbach, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2052–2056
- Tazuma, S., Barnhart, R. L., Reeve, L. E., Tokumo, H. & Holzbach, R. T. (1988) *Am. J. Physiol.* **255**, G745–G751
- Roerdink, F., Regts, J., Van Leeuwen, B. & Scherphof, G. L. (1984) *Biochim. Biophys. Acta* **770**, 195–202
- Braakman, I., Groothuis, G. M. M. & Meijer, D. K. F. (1987) *Hepatology* **7**, 849–855
- Braakman, I. (1988) PhD Thesis, University of Groningen

Received 7 November 1991; accepted 11 December 1991