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ENZYMATIC BASIS FOR THE NON-LINEARITY OF HEPATIC ELIMINATION OF PROPRANOLOL IN THE ISOLATED PERFUSED RAT LIVER

RYOZO ISHIDA,* KAZUYOSHI SUZUKI, YASUHIRO MASUBUCHI, SHIZUO NARIMATSU, SHOICHI FUJITA† and TOKUJI SUZUKI‡

Department of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University 1-33, Yayoi-cho, Inage-ku Chiba 263, Japan

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Abstract—Propranolol (PL) metabolism was studied in the isolated perfused rat liver under single-pass and steady-state conditions. An attempt was made to predict the data observed in the isolated rat liver perfusion at PL infusion rates of 89–1317 nmol/min using the microsomal kinetic parameters obtained in our previous paper (Ishida *et al.*, *Biochem Pharmacol* **43**: 2489–2492, 1992) and the unbound PL fractions in rat liver microsomes and the perfusion medium. The values of kinetic parameters obtained in rat liver microsomes were corrected for the whole liver. Two groups of cytochrome P450 isozymes having high ($K_m < 0.5\ \mu M$)- and low ($K_m > 20\ \mu M$)-affinities participate in the metabolism of PL and sudan III pretreatment induces the low-affinity enzymes rather than the high-affinity enzymes in control rats. Of high-affinity isozyme(s) PL 4-hydroxylase and 7-hydroxylase made a major contribution to the overall activity, while for low-affinity isozymes PL 4-hydroxylase and *N*-desisopropylase did. A non-linear relationship between the PL concentrations entering and leaving the liver was predicted from these corrected kinetic parameters using the venous equilibrium model. The outflow concentrations and the metabolic rates of PL for the predicted curves were over-estimated at higher inflow PL concentrations and under-estimated at higher substrate concentrations, respectively. On the other hand, the prediction for them was successfully carried out for the livers whose intrinsic clearance was altered due to the induction of low-affinity enzymes in PL metabolism by sudan III pretreatment. The outflow rates of 4-hydroxypropranolol showed a downward curvature at lower substrate concentrations, followed a linear rise in the livers from control rats, while the outflow rates of 5- and 7-hydroxypropranolol exhibited their respective limiting values. The outflow rates of 4-hydroxypropranolol and *N*-desisopropylpropranolol were enhanced markedly with increasing the outflow unbound concentration of PL by sudan III pretreatment. These results indicate that non-linear PL first-pass metabolism is due to the saturation of the reactions for the high-affinity enzymes among enzymes engaging in PL ring hydroxylations.

Propranolol (PL§) is a β-adrenergic blocking drug which undergoes extensive first-pass metabolism. A non-linear relationship has been reported between PL dose and its bioavailability or the area under the blood PL concentration–time curve in humans after single [1, 2] and multiple [2–5] oral administration. Dose-dependent bioavailability has also been observed *in vivo* in rats [6, 7] and in the isolated perfused rat liver system [8]. The saturation of PL metabolism has been suggested as the cause of such non-linearity but direct enzymatic evidence has not been provided.

PL has been reported to be metabolized extensively in both humans [9] and animals [10]. Major primary metabolic pathways of PL in the rat are ring hydroxylations at the 4-, 5- and 7-positions of the naphthalene moiety [11], and N-desisopropylation of the aliphatic side chain [12, 13], which are all catalysed by cytochrome P450 (P450). Involvement of multiple isozymes of P450 has been suggested in PL metabolism [14–16]. Furthermore, strong evidence has been reported both in humans [2] and in rats [17] that saturation of the ring oxidation is the principal event responsible for the non-linear PL kinetics. Therefore, the saturation of metabolism suggested for the non-linear hepatic elimination of PL may not mean the saturation of all reactions of its metabolic pathways.

The purpose of this study was to examine if the infusion rate-dependent relationship between inflow and outflow PL concentrations in isolated perfused rat liver preparations can be predicted from *in vitro* enzyme kinetic parameters of PL, and to characterize the reaction(s) responsible for the non-linear first-pass metabolism of PL.

MATERIALS AND METHODS

Chemicals. The sources of reagents were: PL and

* Present address: Central Research Laboratories, Kyorin Pharmaceutical Co. Ltd, 2399-1, Nogi, Nogi-machi, Shimotsuga-gun, Tochigi 329-01, Japan.

† Present address: Department of Toxicology, Faculty of Veterinary Medicine, Hokkaido University, North 18, West 9, Sapporo 060, Japan.

‡ Corresponding author. Tel. (81) 43-251-1111 ext. 2713; FAX (81) 43-255-1574.

§ Abbreviations: PL, propranolol; *N*-ethyl-PL, 1,1-(ethyl-isopropylamino)-3-(1-naphthoxy)-2-propanol; P450, cytochrome P450; *X*-OH-PL, *X*-hydroxypropranolol; ND-PL, *N*-desisopropylpropranolol; C_{in}, inflow concentration; C_{out}, outflow concentration; f_B, unbound fraction in blood.

β-glucuronidase (Type H-1) from the Sigma Chemical Co. (St Louis, MO, U.S.A.); 4-hydroxypropranolol (4-OH-PL) from the Sumitomo Chemical Ind. (Osaka, Japan); *N*-desisopropylpropranolol (ND-PL) from the ICI Pharmaceutical Co. (Macclesfield, U.K.) (all were hydrochlorides); sudan III from the Wako Chemical Co. (Osaka, Japan). 5-OH- and 7-OH-PLs were synthesized as hydrochlorides by the published method [18] with minor modification. *N*-Ethyl-PL [1,1-(ethyl-isopropylamino)-3-(1-naphthoxy)-2-propanol] was synthesized by refluxing a mixture of PL and ethyliodide [19]. Other chemicals were all of analytical grade.

Animals. Male Wistar rats (11 weeks old) were purchased from Takasugi Experimental Animals (Kasukabe, Japan) 1 week prior to use and were kept under our husbandry conditions (12 hr light–dark cycle, 25°). Food (commercially available pellet, the Oriental Yeast Co., Tokyo, Japan) and water were given *ad lib.* A group of rats were given i.p. sudan III (40 mg/kg) dissolved in corn oil for 4 days. The livers of the rats were isolated 24 hr after the last injection.

Preparations of rat liver microsomes and measurement of P450 contents in liver homogenate and microsomes. Liver homogenate samples were prepared by the method of Joly *et al.* [20] and their P450 contents were measured by the method of Omura and Sato [21]. The preparation of liver microsomes and the measurement of their P450 contents were performed according to the method described by Omura and Sato [21]. Microsomal protein was determined by the method of Lowry *et al.* [22].

Isolated rat liver perfusion. The procedures reported by Miller [23] for isolated rat liver perfusion were adopted with minor modifications. Briefly, a perfusion apparatus consisted of a peristaltic pump, a rotating flask with a cannula of oxygen supply to oxygenate the perfusate, a temperature equilibration bath which is also functioning as a humidifier, an air bubble trap, a platform to place an isolated liver, a thermostat and a heater. The perfusate consisted of 25 mM Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 10% (v/v) washed bovine red blood cells, 0.3% (w/v) glucose and 0.4% (w/v) sucrose maintained at 37°. Rats were anesthetized with intraperitoneal pentobarbital. The bile duct was first cannulated, followed by cannulation of the portal vein and hepatic vein. The livers were surgically removed, placed on the platform of the perfusion apparatus, and perfused once through with the perfusing medium via a Harvard infusion pump (Model 975). Perfusates containing PL were infused into the cannula inserted to the portal vein at a flow rate of 15 mL/min after the initial 15 min perfusion without PL.

In the preliminary experiment in which PL infusion of 89 nmol/min was continued for 180 min, stable effluent PL concentrations were attained within 20 min and did not rise during 180 min period. Thus, infusion was carried out at three infusion rates of PL in six liver preparations each from control and sudan III-pretreated rats. Three perfusates containing PL at three different infusion rates among 89, 179, 338, 675, 935 and 1317 nmol/min were passed through the liver sequentially for 40 min each in ascending order. Influent and effluent samples were taken at 40 min and at 31, 34, 37 and 40 min, respectively, from the start of each infusion period. PL and its metabolite concentrations in the samples were determined by the HPLC method as described below. The mean concentration of the effluent samples was taken as the steady-state effluent concentration.

Determination of unbound PL fractions in the perfusate and the microsomal reaction mixture. Equilibrium dialysis was used for the measurement of unbound PL fraction in the supernatant of the perfusate obtained after centrifugation (1200 g × 10 min). The dialysing apparatus was a multiple-cell block each cell of which had two 2.0-mL chambers separated by a semi-permeable membrane (Spectrapor 2, Spectrum Medical Industries, Los Angeles, CA, U.S.A.). One chamber of each cell was filled with 1.0 mL of 25 mM Krebs–Henseleit bicarbonate buffer (pH 7.4) and the other was filled with 1.0 mL of the supernatant samples containing PL. Dialysis was carried out in a shaking water bath at 37°. PL concentrations in both chambers were determined by the HPLC assay as described below. Under these conditions, equilibrium was attained in 16 hr. The unbound PL fraction in the supernatant was calculated by dividing the PL concentration in the dialysate by the total PL concentration in the supernatant. The unbound PL fraction in the perfusate was estimated by multiplying that in the supernatant by a supernatant:perfusate PL concentration ratio.

The unbound PL fraction in the microsomal reaction mixture mentioned in Ref. 16 was determined by the same method as described above. One chamber of the cell was filled with 1.0 mL of

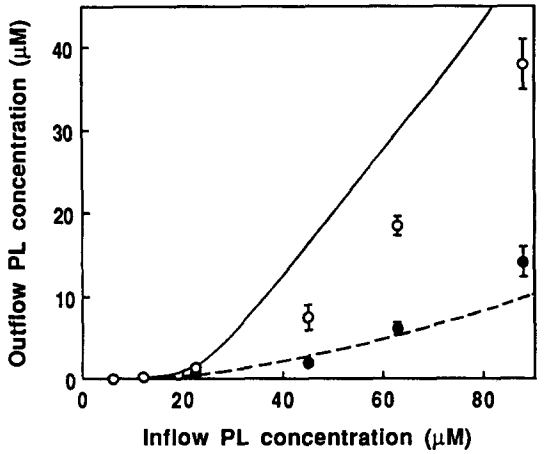

Fig. 1. Plots of the inflow PL concentrations vs the outflow PL concentrations at steady state in the isolated perfused rat liver preparation. Values were observed in the perfused liver preparations from control (○) and sudan III-pretreated (●) rats, respectively. Values represent mean ± SE from three livers. Predicted curves are from the microsomal kinetic parameters of control (——) and sudan III-pretreated (– – –) rats, respectively.

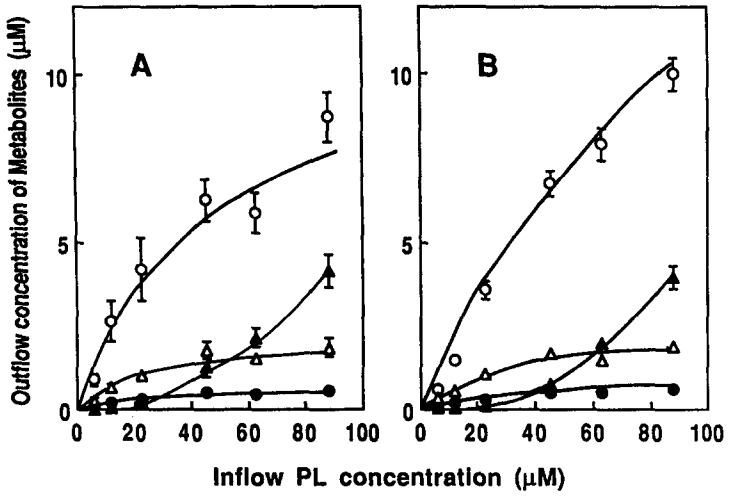

Fig. 2. Plots of the inflow PL concentrations vs the outflow concentrations of primary PL metabolites at steady state in the isolated perfused rat liver preparation. Results were obtained in the perfused liver preparations from (A) control and (B) sudan III-pretreated rats, respectively. Values were obtained for 4-OH (○), 5-OH (●), 7-OH (△) and ND-PL (▲), respectively. Each plot represents mean ± SE from three livers. When no error bar is given, SE is smaller than the symbol used.

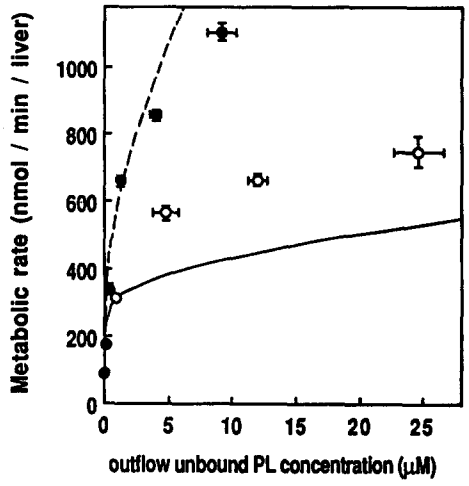

Fig. 3. Relationship between the removal rate of PL and the outflow unbound PL concentration at steady state in the isolated perfused rat liver preparation. Values were observed in the perfused liver preparations from control (○) and sudan III-pretreated (●) rats, respectively. Each plot represents mean ± SE from three livers. Predicted curves are from the microsomal kinetic parameters (corrected for the whole liver) of control (——) and sudan III-pretreated (– – –) rats, respectively.

0.15 M potassium phosphate buffer (pH 7.4) and the other was filled with 1.0 mL of the microsomal reaction mixture containing PL. Dialysis was carried out at 37° for 16 hr. The unbound PL fraction was calculated by dividing the unbound PL concentration in the dialysate by the total PL concentration in the microsomal reaction mixture.

Measurement of PL and its metabolites in the perfusate. PL concentration was assayed by the HPLC method described previously [24] with modifications as follows. One mL of 1 M sodium carbonate buffer (pH 9.8) and 0.1 μg (0.2 mL) of *N*-ethyl-PL as an internal standard were added to each sample (1.0 mL). Ethyl acetate (5 mL) was then added to the alkalinized sample and the mixture was vortexed for 1 min. The sample was centrifuged at 1200 *g* for 10 min and the supernatant was transferred to a tube containing 0.3 mL of 0.01 N HCl. After 1 min vortex mixing, the sample was centrifuged at 1200 *g* for 10 min. The organic layer was discarded by aspiration and the aqueous layer was subjected to HPLC analysis.

For determination of PL metabolites (free and conjugates), the samples (0.5 mL) were incubated at 37° for 2 hr with 0.14 mL of 1.0 M sodium acetate buffer (pH 5.0) containing β-glucuronidase (2000 U)/sulfatase (98 U) of β-glucuronidase (Type H-1), 20 mg ascorbic acid as an antioxidant and 0.1 μg of *N*-ethyl-PL. After hydrolysis, 1 mL of 1 M sodium carbonate buffer (pH 9.8) was added. PL and the PL metabolites were extracted and the HPLC samples were prepared by the method described above. The ring-hydroxylated metabolites of PL in the perfusate existed for the most part as their conjugated forms, and the hydrolysis did not liberate significant quantities of either PL or ND-PL.

An HPLC mobile phase employed was a mixture of acetonitrile, methanol, H_2O, acetic acid in a ratio of 30:25:45:0.2 (by vol.). PL, its metabolites and *N*-ethyl-PL were detected with a spectrofluorometer (excitation and emission wavelengths of 310 and 380 nm, respectively). Under these conditions, typical retention times were: 5-OH-PL, 5.1 min; 4-OH-PL, 5.5 min; 7-OH-PL, 6.3 min; ND-PL, 7.0 min; PL, 9.7 min; and *N*-ethyl-PL, 11.7 min.

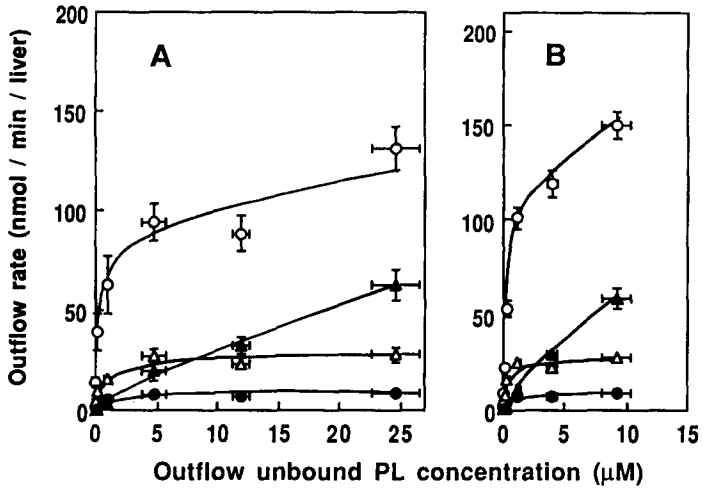

Fig. 4. Relationship between the outflow rate of primary PL metabolites and the outflow unbound PL concentration at steady state in the isolated perfused rat liver preparation. Abbreviations and symbols correspond to those of Fig. 2. Each plot represents mean ± SE from three livers. When no error bar is given, SE is smaller than the symbol used.

RESULTS

Isolated liver perfusion studies

PL was infused for three 40-min periods through isolated rat livers (control) and through isolated livers from sudan III-pretreated rats using a single-pass design. Perfusion with various PL infusion rates (89–1317 nmol/min) resulted in the attainment of steady state for PL and its metabolites by 40 min in each period. In Fig. 1, the steady-state PL concentration in hepatic venous outflow (C_{out}) was plotted against the PL concentration in hepatic inflow (C_{in}). It is apparent from this figure that the relationship between C_{out} and C_{in} is non-linear in these isolated liver preparations. Hepatic availability calculated as C_{out}/C_{in} increased markedly from 0.006 to 0.43 in control rats and from 0.006 to 0.16 in sudan III-pretreated rats with increasing C_{in}.

In Fig. 2, the steady-state concentrations of 4-, 5- and 7-OH-PLs and ND-PL in hepatic venous outflow were plotted against the PL concentration in hepatic inflow. The steady-state concentrations of these metabolites were found to be almost the same for both the livers from control and sudan III-pretreated rats. The concentrations for the ring-hydroxylated metabolites displayed downward curvature as the inflow PL concentration increases. The respective steady-state concentrations of 5- and 7-OH-PLs exhibited their limiting values, but the steady-state concentrations of 4-OH-PL did not. On the other hand, the concentrations of ND-PL curved upward. These observations indicate that a saturable enzyme(s) is involved in the PL ring-oxidations in the range of inflow PL concentrations used.

The metabolic rate (v) of PL under single-pass and steady-state conditions can be obtained by multiplying the hepatic blood flow (Q, 15 mL/min) by the difference between hepatic inflow and outflow PL concentrations:

$$v = Q \times (C_{in} - C_{out}). \quad (1)$$

The effluent unbound concentration was used as the substrate concentration for PL in this study, since the kinetics of hepatic PL elimination were appropriately described with the venous equilibrium model [8, 25], which assumes that the unbound effluent concentration ($f_B C_{out}$) is in equilibrium with the liver cells. The value of f_B (unbound PL fraction) in the hepatic outflow perfusate was calculated to be 0.648 ± 0.042 (mean ± SE, N = 10) from the result of equilibrium dialysis, and this value was constant within the PL concentration range observed in the effluent. The metabolic rate was plotted against the substrate concentration ($f_B C_{out}$ for PL) in the livers from control and sudan III-pretreated rats (Fig. 3). The metabolic rate for sudan III-pretreated rats was increased markedly in higher effluent unbound PL concentrations.

The outflow rates of 4-, 5- and 7-OH-PLs and ND-PL under single-pass and steady-state conditions can be obtained by multiplying the hepatic blood flow by their respective outflow concentrations. The outflow rates for the livers from control and sudan III-pretreated rats were plotted against the substrate concentrations, $f_B C_{out}$ for PL (Fig. 4). The outflow rates of 5- and 7-OH-PLs exhibited their respective limiting values, as the substrate concentration increased. The respective limiting values were not altered by sudan III pretreatment. The outflow rate of 4-OH-PL showed a downward curvature at low substrate concentrations and then changed to a linear rise in the livers from control rats, while a simple linear rise was observed for the outflow rate of ND-PL. The outflow rates of 4-OH-PL and ND-PL were increased markedly by sudan III pretreatment.

Kinetic parameters of PL metabolism for the whole rat liver

Our previous paper [16] reported kinetic parameters of the activities for major metabolic pathways of PL in rat liver microsomes and also in

Table 1. Kinetic parameters for propranolol metabolism for the whole rat liver extrapolated from the microsomal parameters

	Parameter	4-OH		5-OH		7-OH	NDP	
		High	Low	High	Low		High	Low
Control	K_m	0.112 ± 0.019	125 ± 28	0.030 ± 0.008	43.4 ± 16.8	0.117 ± 0.024		70.0 ± 7.7
	V_{max}	202 ± 17	287 ± 29	20.0 ± 1.0	29.3 ± 4.6	109 ± 4		335 ± 13
Sudan III	K_m	0.185 ± 0.041	30.9 ± 3.2	0.055 ± 0.014	21.2 ± 0.4	0.123 ± 0.014	0.673 ± 0.319	29.0 ± 1.4*
	V_{max}	210 ± 37	1080 ± 129†	30.9 ± 2.6	186 ± 26*	163 ± 15	31.6 ± 7.7	1743 ± 135*

Abbreviations: 4-OH, 4-hydroxylation; 5-OH, 5-hydroxylation; 7-OH, 7-hydroxylation; NDP, N-desisopropylation; *High*, high-affinity enzyme(s); *Low*, low-affinity enzyme(s); control and sudan III, microsomes from vehicle control and sudan III-treated rats, respectively.

Each value represents the mean ± SE of three determinations.

The K_m (μM) and V_{max} (nmol/min/whole liver) were calculated from the microsomal parameters obtained in Ref. 16 in the following way: the K_m value was corrected for non-specific binding of PL to microsomes by multiplying 0.642 and 0.590 (unbound fractions in the microsomal reaction mixture for control and sudan III, respectively); the V_{max} value per mg microsomal protein was corrected to obtain the V_{max} per liver using the mean value of the P450 content per g liver (31.5 and 54.3 nmol/g liver for control and sudan III, respectively) and the P450 content per mg microsomal protein (0.741 and 1.14 nmol/mg protein for control and sudan III, respectively) and liver wet weight (12.1 and 13.5 g for control and sudan III, respectively). *, † Significantly different from control values by Student's *t*-test: * $P < 0.05$ and † $P < 0.01$.

those from sudan III-pretreated rats. Sudan III has been shown in rats to induce P450 isozymes which are very similar to those induced by 3-methylcholanthrene with regards to their substrate specificity, electrophoretic pattern and immunochemical metabolic inhibition [26–28].

In this previous study [16], PL 4- and 5-hydroxylations showed biphasic kinetics in both control and sudan III-pretreated rats. PL N-desisopropylation in sudan III-pretreated rats also showed biphasic kinetics, whereas PL 7-hydroxylation in both control and sudan III-pretreated rats and PL N-desisopropylation in control rats exhibited monophasic kinetics. Sudan III pretreatment enhanced PL metabolism at higher PL concentrations except for 7-hydroxylation. The results obtained in this microsomal study [16] indicate that two groups of P450 isozymes having low and high affinities participate in the metabolism of PL, and that sudan III pretreatment induces the low-affinity enzymes rather than the high-affinity enzymes.

In the present study, an attempt was made to predict the data observed in the isolated rat liver perfusion system from the microsomal kinetic parameters and unbound PL fractions in rat liver microsomes and the perfusion medium. V_{max} (nmol/min/g) for the whole liver was obtained by multiplying the specific enzyme activity (nmol/min/mg microsomal protein) by the microsomal protein content (mg microsomal protein/g liver). P450 was used as a marker to correct for microsomal yield [29, 30]. The apparent K_m values obtained from rat liver microsomes from control and sudan III-pretreated rats were corrected for non-specific binding to microsomes by factors of 0.642 and 0.590, respectively, which were virtually constant over the concentration range used. The kinetic parameters thus obtained are listed in Table 1.

Predicted steady-state PL concentrations

The sum (v_{tot}) of metabolic activities for the whole rat liver at a substrate concentration (S) may be estimated by the following equation:

$$v_{tot} = \sum \frac{V_{max(H)i} \times S}{K_{m(H)i} + S} + \sum \frac{V_{max(L)i} \times S}{K_{m(L)i} + S}. \quad (2)$$

Where $V_{max(H)i}$ and $K_{m(H)i}$ are the maximum rate and Michaelis constant of the ith high-affinity enzyme, respectively, and also (L)i denotes the ith low-affinity enzyme. Since the substrate concentration (S) in the liver cells is assumed to equal the unbound PL concentration ($f_B C_{out}$) in the hepatic outflow, Eqns (1) and (2) become:

$$V = Q \times (C_{in} - C_{out}) = \sum \frac{V_{max(H)i} \times f_B C_{out}}{K_{m(H)i} + f_B C_{out}} + \sum \frac{V_{max(L)i} \times f_B C_{out}}{K_{m(L)i} + f_B C_{out}}. \quad (3)$$

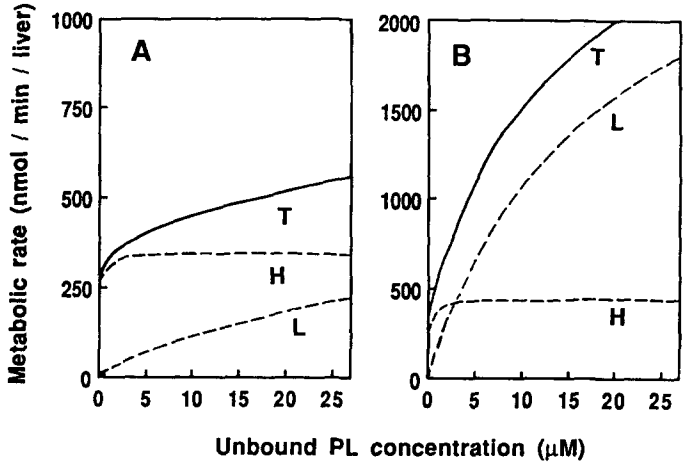

Fig. 5. Simulation of the rate of PL metabolism using pooled rates of high- and low-affinity phases shown in Table 1. Simulation curves (T, solid line) were obtained from the microsomal kinetic parameters (corrected for the whole liver) of (A) control and (B) sudan III-pretreated rats. Curves H and L (dotted lines) indicated pooled rates of high- and low-affinity phases, respectively, and the sum of values on the curves H and L gives the T curve.

By arrangement,

$$C_{out} = C_{in} - 1/Q \times \left(\sum \frac{V_{max(H)i} \times f_B C_{out}}{K_{m(H)i} + f_B C_{out}} + \sum \frac{V_{max(L)i} \times f_B C_{out}}{K_{m(L)i} + f_B C_{out}} \right). \quad (4)$$

Kinetic parameters of PL metabolism for the whole rat liver (Table 1) were used for the prediction of outflow total (bound and unbound) PL concentrations and metabolic rates in the perfused liver at steady state using Eqns (4) and (3), respectively. The predicted concentration curves in the livers from control (solid line) and sudan III-pretreated rats (broken line) are shown in Figs 1 and 3. The outflow total PL concentrations in the livers from control rats were over-estimated at higher inflow PL concentrations (Fig. 1), and the metabolic rates of PL were under-estimated at higher outflow unbound concentrations of PL (Fig. 3). On the other hand, both the predicted curves in the liver from sudan III-pretreated rats fitted well to actual data points.

DISCUSSION

Table 1 shows that kinetic parameters are different depending on the metabolic positions. The total activity of PL metabolism in the whole liver may be estimated by the total sum of the activity of each metabolic reaction (Eqn 2). Figure 5 shows pooled activities for two groups of enzymes classified according to the magnitude (<0.5 and >20 μM) of their Michaelis constants at unbound PL concentrations up to 25 μM. It should be noted that activities of high-affinity enzymes with small K_m values are saturated and their contribution to the total PL metabolism decreases as unbound PL concentration increases, while low-affinity enzymes with greater K_m values gain their contribution. In the present study, the availability of PL in the isolated perfused liver from the control rat was markedly infusion rate-dependent such that a 10-fold increase in inflow PL concentration resulted in about a 50-fold increase in availability (i.e. C_{out}/C_{in}). This remarkable increase in availability is thought to be due to the saturation of enzymes engaging in the high-affinity phase of PL metabolism.

In vivo clearances of drugs have been appropriately predicted from *in vitro* measurements of enzyme parameters (V_{max} and K_m) and of *in vitro* and *in vivo* unbound fractions of drugs [29–32]. The data from the isolated perfused rat liver system (Figs 1 and 3) were predicted from our previous kinetic parameters [16] observed *in vitro* microsomal studies. The unbound PL concentration in the hepatic outflow ($f_B C_{out}$) was used as the substrate concentration in the liver cells which is subject to drug metabolism (venous equilibrium model). The predicted curves for the outflow total PL concentration and the metabolic rates of PL agreed well with the observed values in the livers from sudan III-pretreated rats, whereas the fitting of predicted curves for livers from control rats was not so favorable (Figs 1 and 3). These data in control rats may be due to insufficient recovery of PL metabolism in rat liver microsomes, suggesting another metabolic pathway(s) in the perfused rat liver. On the other hand, the satisfactory fitting of the predicted curve for livers from sudan III-pretreated rats may be interpreted on the assumption that low-affinity enzymes were induced markedly and the contribution of the unknown pathway(s) to the total amount of PL metabolized was negligible, e.g. the pathway(s) was saturable and the activity(ies) was not induced by sudan III pretreatment.

The alteration of the outflow concentrations of 4-, 5- and 7-OH-PLs and ND-PL at steady state by the sudan III pretreatment was not observed, as if sudan III did not induce PL metabolism (Fig. 2). However, the outflow rates of 4-OH-PL and ND-PL at steady state plotted against the substrate concentration ($f_B C_{out}$ for PL) showed that sudan III pretreatment enhanced their respective outflow rates as the substrate concentration increased (Fig. 4). These data suggest the induction of low-affinity enzymes for PL 4-hydroxylation, the activity of which become progressively greater with increasing substrate concentration, and N-desisopropylation. The outflow concentrations of PL metabolites depend upon not only their formation rates from PL but also their elimination rates which may be increased by sudan III pretreatment. Moreover, these further metabolites may be excreted in the bile as reflected partially in a considerable portion of the inflow PL concentration which was not recovered (Figs 1 and 2). Hence the outflow rates of the metabolites do not always indicate their respective formation rates from PL.

In summary, PL metabolism in the isolated perfused rat liver could not be expressed by a single Michaelis–Menten equation, but by the sum of Michaelis–Menten equations, indicating that two groups of P450 isozymes having high and low affinities participate in PL metabolism. From these observations, we conclude that the non-linear PL first-pass metabolism is due to saturation of the reactions for high-affinity enzymes in PL metabolism. Judging from the *in vitro* kinetic studies, these saturable reactions are for high-affinity enzymes involved in PL 4-, 5- and 7-hydroxylations. The saturation of the reactions for low-affinity enzymes involving 4-hydroxylation and N-desisopropylation does not seem to occur in the infusion-rate range used.

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