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Metabolism of an Anionic Fluorescent Dye, 1-Anilino-8-naphthalene Sulfonate (ANS) by Rat Liver Microsomes

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The present study was designed to examine the metabolism of 1-anilino-8-naphthalene sulfonate (ANS), an anionic compound which is transported into liver via "multispecific organic anion transporter", with rat hepatic microsomes. TLC analysis indicated that the fluorescent metabolites were not produced to a measurable extent, which made it possible to assess the ANS metabolism by measuring the fluorescence disappearance. The metabolism of ANS was remarkably inhibited by the presence of SKF-525A as well as by the substitution of O_2 by CO gas. ANS metabolism by microsomes also required NADPH as a cofactor. These results indicated that the microsomal monooxygenase system might be mainly responsible for the ANS metabolism. The maximum velocity (V_{max}) and Michaelis constant (K_m) were calculated to be 4.3 $\pm$0.2 nmol/min/mg protein and 42.1$\pm$2.0 μM, respectively. Assuming that 1 g of liver contains 32 mg of microsomal protein, the V_{max} value was extrapolated to that per g of liver (V_{max}'). The intrinsic metabolic clearance (CL_{int}) under linear conditions calculated from this *in vitro* metabolic study was 3.3 ml/min/g liver, being comparable with that (3.0 ml/min/g liver) calculated by analyzing the in vivo plasma disappearance curve in a previous study. Furthermore, the effects of other organic anions on the metabolism of ANS were examined. Bromophenolblue (BPB) and rose bengal (RB) competitively inhibited the metabolism of ANS, while BSP inhibited it only slightly. The inhibition constant (K_i) of BPB (6 μM) was much smaller than that of RB (200 μM). In conclusion, the microsomal monooxygenase system plays a major role in the metabolism of ANS, and other unmetabolizable organic anions (BPB and RB) compete for this metabolism.

Key words : 1-Anilino-8-naphthalene sulfonate, Metabolism, Microsomes, Intrinsic clearance, Competitive inhibition

INTRODUCTION

Recently, we have clarified the hepatic transport of 1-anilino-8-naphthalene sulfonate (ANS), an anionic fluorescent dye, on the basis of inhibition studies or known transport characteristics (Chung *et al.*, 1990a). ANS was taken up by the liver, being mediated by a common transport carrier with the other organic anions such as BSP, RB, and BPB. Moreover, we determined the dose-dependent hepatic transport of ANS, measuring the time-profiles of its plasma disappearance at various doses (3~100 μmol/kg) (Chung *et al.*, 1990b). We have confirmed the saturability in the sequestration process assessed from the analysis of the plasma disappearance curve. After i.v. bolus administration, ANS was mainly taken up by the liver within 10 min.

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Organic anions are rapidly and selectively taken up from circulation into hepatocytes via more than one uptake system (Elferink *et al.*, 1995). Examples of such systems are the Na^+-coupled secondary active transport and Na^+-independent uptake for bile acids (Anwer *et al.*, 1978a), Na^+-dependent uptake for long-chain free fatty acids (Stremmel *et al.*, 1986), and Na^+-independent uptake for nonbile acid cholephils such as bilirubin (Paumgartner *et al.*, 1976), bromosulfophthalein (BSP) (Laperche *et al.*, 1981; Schwenk *et al.*, 1976; Wolkoff *et al.*, 1987), and indocyanine green (ICG) (Scharschmidt *et al.*, 1975). Na^+-dependent bile acid uptake system has been dubbed the "multispecific Na^+-dependent bile acid transport system" (Zimmerli *et al.*, 1989; Petzinger *et al.*, 1984; Petzinger *et al.*, 1989; Ziegler *et al.*, 1985). Furthermore, Na^+-independent bile acid uptake and organic anion transport proved to be mediated by a common transport carrier (Anwer *et al., 1978b; Laperche et al.*, 1981), which is designated as a "multispecific organic anion transporter" (Meier 1988; Blom *et al.*, 1981; Tsuji *et al.*, 1986). On the

other hand, organic anions can be classified into two groups in terms of ATP dependency in hepatic uptake, i.e., via active transport or facilitated diffusion (Yamazaki *et al.*, 1992). Dibromosulfophthalein (DBSP) and benzylpenicillin belong to the former group, whereas BSP, rose bengal (RB), and bromophenol blue (BPB) belong to the latter (Yamazaki *et al.*, 1992). ANS, a fluorescent model compound for studing hepatic transport of anionic drugs, appears to be taken up via "multispecific organic anion transporter" by the liver (Chung *et al.*, 1990a). The amount of the parent ANS excreted into the bile was less than 2% (Chung *et al.*, 1990b). ANS excretion into the urine was also negligible, suggesting that the disposition of ANS in the whole body might be attributed mainly to the metabolism in liver. However, direct demonstration of ANS metabolism in the liver has not been obtained yet.

In the present study, we examined whether the saturable metabolism of ANS occurred in hepatocytes, using rat hepatic microsomes. Furthermore, we attempted to reconstruct the sequestration clearance *in vivo*, based on the metabolic parameters determined *in vitro* with rat hepatic microsomes.

METERIALS AND METHODS

Meterials

1-Anilino-8-naphthalene sulfonate (sodium salt) and bromophenol blue were purchased from Tokyo Chemical Co. (Tokyo, Japan). Rose bengal was purchased from Wako Pure Chemical Co. (Tokyo, Japan). Bromosulfophthalein and SKF-525A were purchased from Daiichi Chemical Co. (Tokyo, Japan). NADPH and 8-glucuronidase/arylsulfatase were purchased from Boehringer Mannheim Gmbh (W. Germany). All other reagents were commercial products and of analytical grade. Male S.D. rats (Samuk Animal Farm, Kyunggi-Do, Korea) weighing 240~280 g were used.

Metabolism of ANS by microsomes

Rat hepatic microsomes was isolated as described previously (Chung *et al.*, 1990a). Fifty mM Tris-HCl buffer (pH 7.4) containing 1 mg/ml microsomal protein, 10 mM $MgCl_2$, and ANS of various concentrations (5~200 μM) were used as a reaction mixture. After preincubation for 5 min at 37°C, an aliquot of NADPH was added to the incubation mixture to start the metabolic reaction (initial NADPH concentration: 1 mM). An aliquot of the incubation mixture (100 μl) was taken at given times (0, 2, 5, 10, 20, 30 min) and added to 3.5 ml of ethanol to stop the reaction. After centrifugation, ANS in the supernatant was fluorometrically determined at 470 nm (excited at 410 nm), using an Hitachi MPF-4 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). The experiments were also performed under the following conditions to examine whether ANS is metabolized by the microsomal monooxygenase system; 1) the incubation medium bubbled with CO gas, 2) the incubation medium containing heat-denatured microsomes, 3) the incubation medium in the absence of NADPH, 4) the incubation medium in the presence of SKF-525A, and 5) the incubation on ice. During 5 min of preincubation without NADPH, the fluorescence intensity of ANS remained unchanged, suggesting that ANS was not metabolized during this period.

Analysis by thin layer chromatography (TLC)

To determine whether a fluorescent metabolite was produced by hepatic microsomes, we separated the fluorescent molecules in the incubation medium by TLC (Chung *et al.*, 1990a). A hundred μl of the incubation medium was added to the equivalent volume of ethanol, followed by centrifugation (1000× g, 10 min), and then 150 μl of the supernatant was applied to a TLC plate (silica gel G, 20×20 cm). The TLC plate was developed in a mixture of chloroform/methanol/water (65:25:4, v/v). Every 1.5 cm length of the TLC plate was scraped and extracted with 4 ml of ethanol. The ANS in the supernatant was fluorometrically determined at 470 nm (excited at 410 nm).

Effect of organic anions on the metabolism of ANS

We determined the effect of other organic anions (RB, BPB and BSP) on the ANS metabolism by hepatic microsomes. After 5 min of preincubation at 37°C, an aliquot of NADPH (initial concentration: 1 mM) was added to the incubation mixture. An aliquot of the incubation mixture (100 μl) was taken at given times and added to 3.5 ml ethanol. The initial concentration of ANS in the incubation mixture was kept at 20 μM, while those of other organic anions ranged from 10 to 200 μM. The ANS concentrations in the incubation mixture were fluorometrically determined as described above. The quenching effects induced by organic anions on the fluorescence intensity of ANS were checked, and the fluorescent intensities were corrected for the "inner-filter" effect, if necessary (Sugiyama *et al.*, 1983).

Kinetic analysis

The initial velocity (v) of the ANS metabolism was calculated from the initial decay slope of the fluorescence disappearance curve by linear regression (within 5 min). A double reciprocal linear transformation of the data (Lineweaver-Burk plot) yielded a straight line. The data for the ANS metabolism were fitted to the Michaelis-Menten equation, and the enzymatic parameters (K_m, V_{max}) were calculated with a nonlinear least

squares method (Yamaoka *et al.*, 1981). Furthermore, we determined the intrinsic clearance for ANS metabolism, which is calculated as V_{max}/K_m after extrapolating the V_{max} value to that per gram of liver (V_{max}'), considering that 1 gram of liver contains 32 mg of microsomal proteins (Lin *et al.*, 1980; Joly *et al.*, 1975).

RESULTS AND DISCUSSION

ANS metabolism by microsomes

We previously detected the fluorescent metabolite in bile samples using TLC analysis (Chung *et al.*, 1990a). The amount of fluorescent metabolite excreted into the bile was, however, at most 2% of the dose. This metabolite was not detected in the plasma. These findings suggested that the metabolism of ANS to the fluorescent molecules was negligible. In the present study, we examined whether fluorescent metabolite was produced after 30 min-incubation with hepatic microsomes. The fluorescence attributable to only parent ANS (Rf=0.45) was detected, suggesting that the metabolism of ANS to the fluorescent metabolites might be negligible. Consequently, the ANS metabolism in hepatic microsomes was assessed by directly measuring the fluorescence disappearance (Fig. 1).

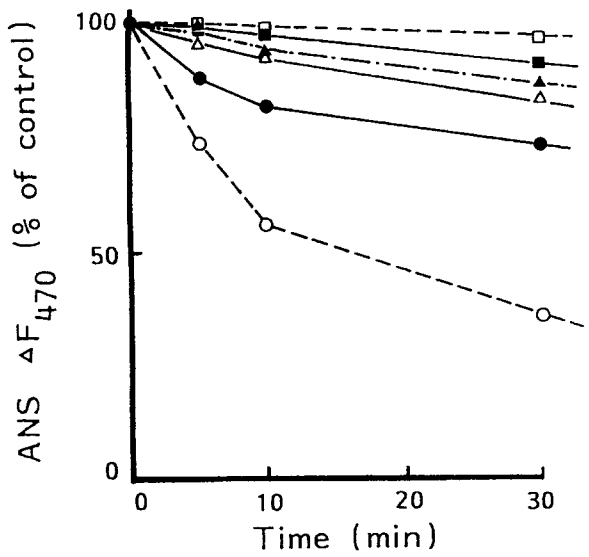

Fig. 1. The time profiles of the disappearance of ANS fluorescence in rat hepatic microsomes. After preincubation for 5 min at 37°C, an aliquot of NADPH (initial concentration: 1 mM) was added to the incubation mixture containing 10 μM ANS and 1 mg protein/ml hepatic microsomes. An aliquot of the incubation mixture (100 μl) was taken at given times and added to 3.5 ml of ethanol to stop the reaction. ANS concentration was fluorometrically determined at 470 nm (excited at 410 nm). The fluorescence intensity was expressed as the percentage of that at time zero. Each point represents the average value obtained using two different preparations each of those was prepared using 3 rats. Key; (○), control; (●), incubation in the presence of 1 mM SKF-525A; (△), incubation at 4°C; (▲), incubation with bubbled CO gas; (■), incubation with heat-denatured microsomes; (□), incubation in the absence of NADPH.

Fig. 1 shows the time courses of the disappearance of ANS fluorescence. The ANS metabolism was markedly inhibited by SKF-525A as well as by the substitution of O_2 by CO gas, both of which are well known to inhibit oxidation by the hepatic microsomal monooxygenase system(Conney *et al.*, 1968; Schenkman 1972; Jenner 1972). ANS metabolism also required NADPH as a cofactor and showed a temperature dependence (Fig. 1). These results indicate that the microsomal monooxygenase system might be mainly responsible for the ANS metabolism. The incubation mixture obtained after 30 min-incubation in a control experiment was further treated with 6-glucuronidase/arylsulfatase, but the fluorescent intensity was unchanged, suggesting that the conjugation of ANS and/or its oxidative fluorescent metabolites was negligible.

Kinetic analysis

When the initial velocity of ANS metabolism assessed from the initial decay slope of the fluorescence disappearance curve was plotted against its initial concentration, a hyperbolic curve was obtained. A Lineweaver-Burk plot yields a straight line (Fig. 2). The maximum velocity (V_{max}) and the Michaelis constant (K_m) were calculated to be 4.3±0.2 nmol/min/mg protein and 42.1±2.0 μM, respectively. Considering that 1 gram of liver contains 32 mg microsomal of protein (Lin *et al.*, 1980; Joly *et al.*, 1975), the Vmax value was extrapolated to that per gram of liver (V_{max}'). The intrinsic clearance (CLint) under linear conditions is given by the ratio of V_{max}' to K_m value and was calculated to be 3.3 ml/min/g liver. We previously determined the intrinsic clearance of ANS representing the sequestration process by analyzing the plasma disappearance curve after iv administrations. In that study, the plasma disappearance curves of ANS at various doses were simultaneously analyzed, based on a nonlinear kinetic model which incorporated the saturable binding to intracellular proteins and the saturable elimination in hepatocytes. This analysis based on the *in vivo* data gave the V_{max} and K_m values to be 30 nmol/min/g liver and 10 μM, respectively. The CL_{int} value (V_{max}/K_m) thus was calculated to be 3 ml/min/g liver from the *in vivo* data and was comparable to that (3.3 ml/min/g liver) determined with hepatic microsomes. These results may suggest that the sequestration process determined from the *in vivo* experiment mainly reflects the metabolic process by the microsomal monooxygenase system. Such an extrapolation of intrinsic clearance from *in vitro* to *in vivo* was also successful in the metabolism of several drugs (Lin *et al.*, 1980; Joly *et al.*, 1975). However,

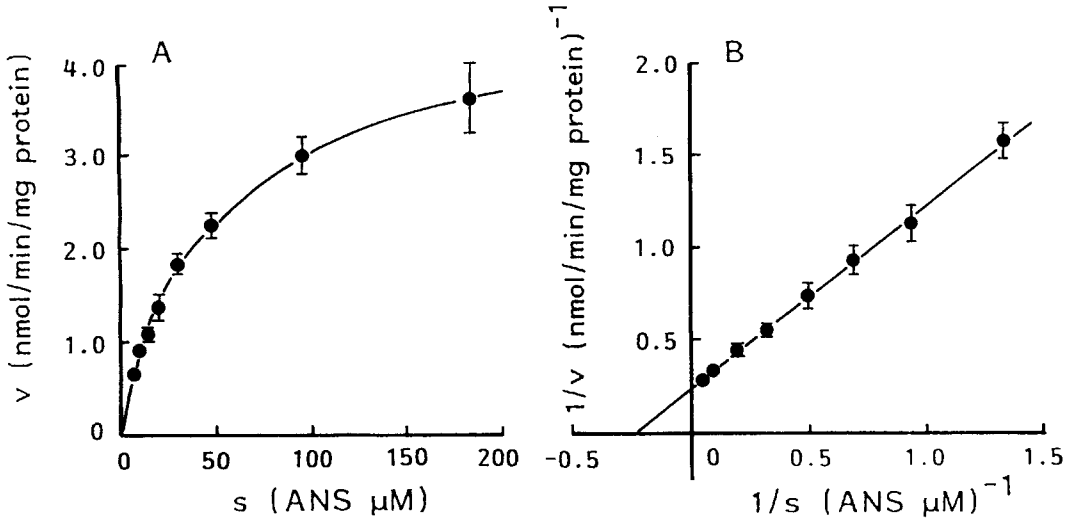

Fig. 2. Lineweaver-Burk plot of ANS metabolism by hepatic microsomes. The initial velocity of ANS metabolism was calculated by measuring the decrease in ANS amount from the incubation mixture (0~5 min). The solid line represents the best fit to the data based on the Michaelis-Menten equation. Each point represents the mean±S.E. of four preparations.

comparing the K_m and V_{max} values individually, these parameters determined from the *in vitro* study were several times larger than those determined from the *in vivo* study. In the present study, the discrepancy of the K_m values between the *in vivo* and *in vitro* might be partly due to the binding of ANS to microsomes. To calculate the *in vivo* parameters, we used the unbound fraction of ANS in the liver, determined using the equilibrium dialysis with diluted cytosol (Chung *et al.*, 1990a). Possible errors in estimating the unbound fraction in the liver by such a method might also cause the discrepancy between the K_m and V_{max} values in the *in vivo* and *in vitro* studies.

Inhibitory effect of organic anions on the ANS metabolism

We determined the effects of other organic anions (BPB, BSP, or RB) on the metabolism of ANS (Fig. 3). BPB and RB markedly inhibited the metabolism of ANS in a concentration dependent manner, while BSP only slightly inhibited the metabolism of ANS. The inhibitory effect of BPB on the metabolism of ANS was larger than that of RB. Fig. 4 shows the Lineweaver-Burk plots of ANS metabolism in the presence and absence of other organic anions (BPB or RB). Such analysis indicated that these organic anions competitively inhibited ANS metabolism. The kinetic parameters are summarized in Table I, indicating that the inhibition constant (K_i) for BPB was much smaller than that for RB.

Recently, we have demonstrated that pulse administration of an excess amount of other organic anions (BSP, BPB) at 10 min after iv administration of ANS induced transient increase in the ANS plasma concentration (Chung *et al.*, 1990b). This phenomenon has been called "*in vivo* counter transport" (Scharschmidt *et al.*, 1975). The kinetic analysis indicated that this "*in vivo* counter transport" phenomena may be explained not only by the inhibition of the uptake via the carrier on the plasma membrane but also by the displacement

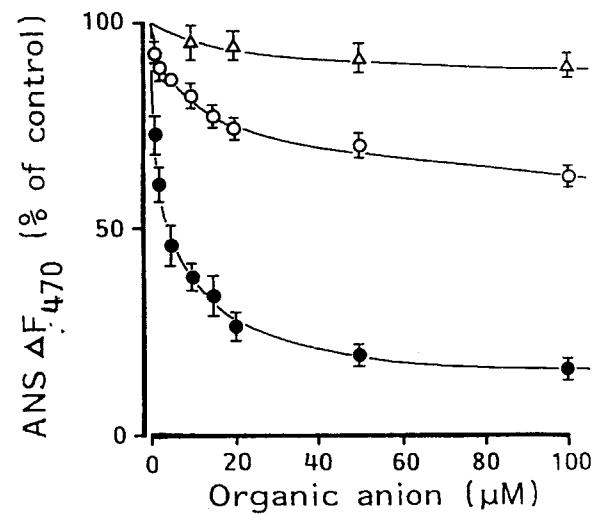

Fig. 3. Effects of organic anions (BPB, BSP, or RB) on ANS metabolism by hepatic microsomes, determined by the fluorescence disappearance of ANS. After preincubation for 5 min at 37°C, NADPH was added to the incubation mixture containing 20 μM ANS and 0~100 μM organic anion and 1 mg protein/ml hepatic microsomes. An aliquot (100 μl) of the incubation mixture was taken at 5 min and added to 3.5 ml ethanol. After centrifugation, ANS in the supernatant was fluorometrically determined at 470 nm (excited at 410 nm). The fluorescence disappearance rate was expressed as the percentage of that in the absence of organic anions. Each point represents the mean±S.E. of three different preparations. Keys: (●)+BPB; (○)+RB; (△)+BSP.

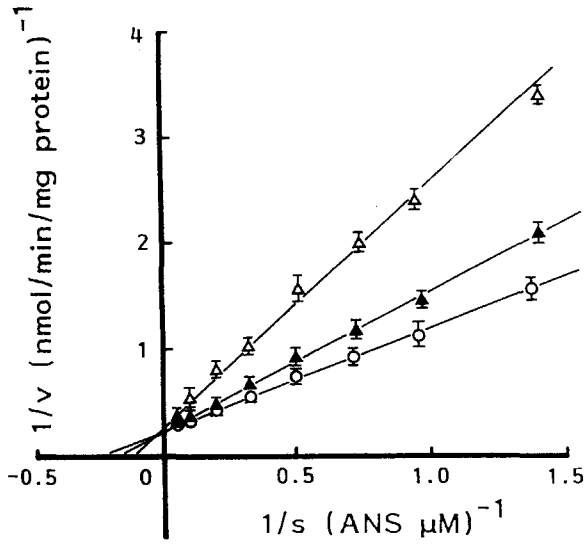

Fig. 4. Lineweaver-Burk plots of ANS metabolism by hepatic microsomes in the absence and presence of other organic anions. Each point represents the mean±S.E. of four different preparations. Keys; (●), control (20 μM ANS); (▲)+50 μM RB; (△)+5 μM BPB.

Table I. Effect of BPB or RB on the metabolic parameters of ANS by rat liver microsomes[a)]

	V_{max}[b)] (nmol/min /mg protein)	$K_{m,app}$[b)] (μM)	V_{max}'[c)] (nmol/min /g liver)	Ki[d)] (μM)
Control	4.3±0.2	42.1±2.0	138±6.0	
+RB (50 μM)	4.2±0.2	55.5±2.8	136±5.0	196±63.0
+BPB (5 μM)	3.6±0.4	82.4±8.5	116±12.0	6.0±1.3

[a)]Mean±S.E. of four different preparations.
[b)]V_{max} and $K_{m,app}$ values were calculated by nonlinear least squares method (Yamaoka *et al.*, 1981).
[c)]V_{max}' value per gram liver. See details in the text.
[d)]Calculated assuming competitive inhibition.

of ANS binding from its intracellular binding sites (Chung *et al.*, 1990b). It is also possible that the inhibition of ANS metabolism by other organic anions increases the intracellular ANS concentration, leading to the delay of its plasma disappearance by enhancing its back diffusion to the plasma. Therefore, the "*in vivo* counter transport" phenomenon induced by these organic anions might be affected also by the inhibition of ANS metabolism.

CONCLUSION

The metabolism of ANS was inhibited by SKF-525A as well as by the substitution of O_2 by CO gas. ANS metabolism also required NADPH as a cofactor. These results suggest that the microsomal monooxygenase system plays a major role in the metabolism of an anionic fluorescent dye, ANS, in the rat liver. The other amphiphatic unmetabolizable organic anions, BPB and RB competitively inhibited the ANS metabolism by microsomes.

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REFERENCES CITED

Anwer, M. S. and Hegner, D., Effect of Na^+ on bile acid uptake by isolated rat hepatocytes; evidence for a heterogenous system. *Hoppe-Seyler's Z. Physiol. Chem.*, 359, 181-192 (1978a).

Anwer, M. S. and Hegner, D., Effect of organic anions on bile acid uptake by isolated rat hepatocytes. *Hoppe-Seyler's Z. Physiol. Chem.*, 359, 1027-1030 (1978b).

Blom, A., Keulemans, K. and Meijer, D. K. F., Transport of dibromosulfophthalein by isolated rat hepatocytes. *Biochem. Pharmacol.*, 30, 1809-1816 (1981).

Chung, Y. B., Miyauchi, S., Sugiyama, Y., Harashima, H., Iga, T. and Hanano, M., Kinetic analysis of the dose-dependent hepatic handling of 1-anilino-8-naphthalene sulfonate in rats. *J. Pharmacokin. Biopharm.*, 18, 313-333 (1990a).

Chung, Y. B., Miyauchi, S., Sugiyama, Y., Harashima, H., Iga, T. and Hanano, M., Effect of various organic anions on the plasma disappearance of 1-anilono-8-naphthalene sulfonate (ANS). *J. Hepatology*, 11, 240-251 (1990b)

Conney, A. H., Levin, W., Ikeda, M. and Kuntzman, R., Inhibitory effect of carbon monoxide on the hydroxylation of testosterone by rat liver microsomes. *J. Biol. Chem.*, 234, 3912-3915 (1968).

Elferink, R. P. J., Meijer, D. K. F., Kuipers, F., Jansen, P. L. M., Groen, A. K. and Groothuis G. M. M., Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. *Biochim. Biophys. Acta*, 1241, 215-268 (1995).

Jenner, S. and Netter, K.J., On the inhibition of microsomal drug metabolism by SKF *Biochem. Pharmacol.* 21, 1921-1927 (1972).

Joly, J.-G., Doyon, C. and Pesant, Y., Cytochrome P-450 measurement in rat liver homogenate and microsomes. Its use for correction of microsomal losses incurred by differential centrifugation. *Drug. Metab. Disposit.*, 3, 577-586 (1975).

Laperche, Y., Preaux, A. M. and Berthelot, P., Two systems are involved in the sulfobromophthalein uptake by rat liver cells: one is shared with bile salts. *Biochem. Pharmacol.* 30, 1333-1336 (1981).

Lin, J. H., Sugiyama, Y., Awazu, S. and Hanano, M., Kinetic studies on the deethylation of ethoxybenzamide. A comparative study with isolated hepatocytes

and liver microsomes of rat. *Biochem. Pharmacol.*, 29, 2825-2830 (1980).

Meier, P. J., Transport polarity of hepatocytes. *Semin. Liver Dis.*, 8, 293-307 (1988).

Paumgartner, G. and Reichen, J., Kinetics of hepatic uptake of unconjugated bilirubin. *Clin. Sci. Mol. Med.*, 51, 169-176 (1976).

Petzinger, E. and Frimmer, M., Driving forces in hepatocellular uptake of phalloidin and cholate. *Biochim. Biophys. Acta.*, 778, 539-548 (1984).

Petzinger, E., Muller, N., Folimann, W., Deutscher, J. and Kinne, R.K.H., Uptake of bumetanide into isolated rat hepatocytes and primary liver cell cultures. *Am. J. Physiol.* 256 (Gastrointest. Liver Physiol. 19), G78-G86 (1989).

Scharschmidt, B.F., Waggondr, J.G. and Berk, P.D., Hepatic organic anion uptake in the rat. *J. Clin. Invest.*, 56, 1280-1292 (1975).

Schenkman, J. B., Wilson, B. J. and Cinti, D. L., Dimethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A)-*in vivo* and *in vitro* effects of metabolism by rat liver microsomes-formation of an oxygenated complex. *Biochem. Pharmacol.*, 21, 2373-2383 (1972).

Schwenk, M., Burr, R., Schwarz, L. and Pfaff, E., Uptake of sulfobromophthalein by isolated rat liver cells. *Eur. J. Biochem.*, 64, 189-197 (1976).

Stremmel, W., Strohmeyer, S. and Berk, P.D., Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc. Natl. Acad. Sci. USA,* 83, 3584-3594 (1986).

Sugiyama, Y., Kimura, S., Lin, J. H., Izuma, M., Awazu, S. and Hanano, M., Effect of organic anions on the uptake of I-anilino-8-naphthalene sulfonate by isolated liver cells. *J. Pharm. Sci.*, 72, 871-876 (1983).

Tsuji, A., Terasaki, T., Takanosu, T., Tamai, I. and Nakashima, E., Uptake of Benzylpenicillin, cefpiramide, and cefazolin by freshly prepared rat hepatocytes. *Biochem. Pharmacol.*, 35, 151-158 (1986).

Wolkoff, A.W., Samuelson, A.C., Johansen, K.L., Nakata, R., Withers, D.M. and Sosiak, A., Influence of Cl^- on organic anion transport in short-term cultured rat hepatocytes and isolated rat liver. *J. Clin. Invest.*, 79, 1259-1268 (1987).

Yamaoka, K., Tanigawara, Y., Nakagawa, Y. and Uno, T., A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.*, 4, 879-885 (1981).

Yamazaki, M., Suzuki, H., Sugiyama, Y., Iga, T. and Hanano, M., Uptake of organic anions by isolated rat hepatocytes: a classification in terms of ATP-dependency. *J. Hepatol.*, 14, 41-47 (1992).

Ziegler, K., Frimmer, M., Kesseler, H., Damm, J., Eiermann, V., Koll, S. and Zarbock, J., Modified somatostatins as inhibitors of a multispecific transport system for bile acids and phallotoxins in isolated hepatocytes. *Biochim. Biophys. Acta.*, 845, 86-93 (1985).

Zimmerli, B., Valantinas, J. and Meier, P.J., Multispecificity of Na^+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. *J. Pharmacol. Exp. Ther.*, 250, 301-308 (1989).