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Kinetic Analysis about the Bidirectional Transport of 1-Anilino-8-naphthalene Sulfonate (ANS) by Isolated Rat Hepatocytes

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The purpose of the present study was to investigate the bidirectional transport of 1-anilino-8-naphthalene sulfonate (ANS) using isolated rat hepatocytes. The initial uptake rate of ANS by isolated hepatocytes was determined. The uptake process of ANS was saturable, with a K_m of 29.1±3.2 μM and V_{max} of 2.9±0.1 mmol/min/mg protein. Subsequently, the initial efflux rate of ANS from isolated hepatocytes was determined by resuspending preloaded cells to 3.0% (w/v) BSA buffer. The efflux process for total ANS revealed a little saturability. The mean value of the efflux clearance was 2.2±0.1 μL/min/mg protein. The efflux rate of ANS from hepatocytes was markedly decreased at 4°C, indicating that the apparent efflux of ANS might not be attributed to the release of ANS bound to the cell surface, but to the efflux of ANS from intracellular space. The efflux clearance was furthermore corrected for the unbound intracellular ANS concentration on the basis of its binding parameters to cytosol. The relation between efflux rate and unbound ANS concentration was fitted well to the Michaelis-Menten equation with a saturable and a nonsaturable components. The V_{max} and K_m values were 0.54 mmol/min/mg protein, and 10.0 μM, respectively. Based on the comparison of the ratios of V_{max} to K_m (V_{max}/K_m) corresponding to the transport clearance, the influx clearance was two times higher than the efflux clearance. Together with our preliminary studies that ATP suppression in hepatocytes substantially inhibited ANS influx rate, we concluded that the hepatic uptake of ANS is actively taken up into hepatocytes via the carrier mediated transport system.

Key words: 1-Anilino-8-naphthalene sulfonate (ANS), Hepatocytes, Uptake, Efflux, Bidirectional transport

INTRODUCTION

The fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), has been used to quantitate the binding of organic anions with the binding proteins (Cheng *et al.*, 1978) by utilizing the fluorescent change of ANS due to the binding displacement by other ligands. Previously, we demonstrated that ANS was taken up into hepatocytes via a carrier mediated transport system, which was common to other organic anions, such as bromosulfophthalein (BSP), rose bengal (RB), indocyanine green (ICG) (Chung *et al.*, 1990a). 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). After *i.v.* bolus administration, ANS was mainly taken up by the liver within 10 min. ANS excretion into the urine was also negligible, suggesting that the disposition of ANS in the whole body might be attributed mainly to the uptake into the liver (Chung *et al.*, 1990b).

Organic anion uptake and Na^+-independent bile acid transport proved to be mediated by a common transport carrier (Yamazaki *et al.*, 1996; Kusuhara *et al.*, 1998; 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, RB, and BPB belong to the latter (Yamazaki *et al.*, 1992). ANS, a fluorescent model compound for studing hepatic transport of anionic

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drugs appears to be taken up via "multispecific organic anion transporter" by the liver (Chung *et al.*, 1990a).

Binding to cytosolic protein may partially explain hepatic cellular accumulation of ANS (Chung *et al.*, 1990b). Yamazaki *et al.* (1989) previously showed that the initial fluorescence change of ANS, considered to represent the uptake, was affected little by several metabolic inhibitors. On the contrary, we recently found that 30 M rotenone reduced the cellular accumulation of ANS to a half by directly determining the ANS taken up by the isolated hepatocytes, suggesting that the uptake process of ANS is energy-dependent.

In the present study, therefore, the bidirectional transport of ANS by isolated rat hepatocytes was characterized in order to explain its concentrated uptake.

MATERIALS AND METHODS

Materials

^{14}C-inulin and ^{3}H-H_2O were purchased from New England Nuclear Corp. (Boston, MA). ANS was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (Fraction V) (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were commercial products and of analytical grade. Male S.D. rats (Sam Tac, Kyunggi-Do, Korea) weighing 250-300 g on a normal laboratory diet were used throughout.

Preparation of isolated hepatocytes

Hepatocytes were isolated from male S.D. rats (240-280 g) by the procedure of Iga *et al.* (1979). After isolation, hepatocytes were suspended (1.7×10^6 cell/mL) at 0°C in the albumin-free Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.4). The viability of isolated cells was determined by 0.4% trypan blue exclusion test. The value obtained usually ranged from 95% to 98%.

Uptake of ANS by isolated hepatocytes

To determine the uptake rate of ANS, isolated hepatocytes (1.7×10^6 cells/mL) were incubated at 37°C with various concentrations of ANS. After 5 min of preincubation of the cells at 37°C, an aliquot (50 μL) of ANS was added to start uptake. Initial concentrations of ANS ranged from 5 μM to 70 μM. The incubation medium (albumin-free) in the uptake experiment contained 137 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 10 mM Hepes, and 5 mM glucose (pH 7.4).

An aliquot of the cell suspension (100 μL) was taken at the indicated times (20, 40, 60, 90, 120, and 300 sec), and laid on top of a two-phase system in microfuge tubes. The bottom phase consisted of glycerol (100 μL). This was overlaid with 500 μL of silicone mineral oil (density 1.015). The tubes were then centrifuged for 20 s in a table-top microfuge (Beckman Instruments, Fullerton, CA, USA). Three mL of ethanol was added to the glycerol solution. After centrifugation of the mixtures at 3,000 rpm for 15 min at 4°C, ANS amounts in the organic phase were fluorometrically determined at 480 nm (excited at 400 nm) as previously reported (Chung *et al.*, 1990a). Amounts taken up by hepatocytes were corrected for the adherent film (2.2 μL/mg protein). Adherent water volume was determined with [^{14}C] inulin and $^{3}H_2O$ (Yamazaki *et al.*, 1992). Protein was determined by protein assay kits (Bio-Rad Co. Ltd.). Bovine serum albumin was the standard.

Efflux of ANS by isolated hepatocytes

After 5 min period preincubation of the cell suspension (7×10^6 cells/mL) at 37°C, an aliquot of ANS was added, and the incubation was continued during 3 min to accumulate ANS into hepatocytes. After 3 min period incubation, 2.5 mL of ANS preloaded cells were resuspended into 2.5 mL of prewarmed buffer (at 37°C) containing 3% (w/v) BSA. We preliminarly confirmed that ANS was taken up by hepatocytes in 3% (w/v) BSA buffer to a negligible extent, suggesting that the reuptake of ANS released to the BSA containing buffer might be minimal. An aliquot of cell suspension was taken at designated times (0, 20, 40, 60, 90, and 120 sec), and laid on the top of the two-phase system in microfuge tube. The amount of ANS taken up by the hepatocytes was fluorometrically determined as described above.

The amounts of ANS taken up by hepatocytes were corrected for the adherent water film (2.2 μL/mg protein) and were also expressed as per mg protein. The intracellular concentration of ANS was calculated by dividing the amount in the hepatocytes by the intracellular space volume (cellular volume (V_{cell}): 5.2 μL/mg protein). The adherent water volume and intracellular volume were determined using ^{14}C-inulin and $^{3}H_20$, respectively. The protein concentration was determined by protein assay kits (Bio-Rad Co. Ltd.) with BSA as a standard.

Determination of the uptake and efflux permeability clearance

The initial uptake rate of ANS (v_{inf}) was obtained by the regression of the linear portion of uptake time course (within 1 min). The relation between initial uptake rate (v_{inf}) and the initial ANS concentration in the medium (s) was plotted as Eadie-Hofstee plot. This plot revealed the straight line, and therefore we fitted the uptake data to a Michaelis-Menten equation with a saturable component.

$$v_{inf}=\frac{V_{max}[S]}{K_{max}+[S]} \quad (1)$$

where V_{max} and K_m represent the maximum uptake velocity

(mmol/min/mg protein) and Michaelis-Menten constant (μM), respectively. The V_{max} and K_m values were calculated with a nonlinear least squares method.

The initial efflux rate of ANS (v_{eff}) was calculated by regression analysis of the linear portion of efflux time course (within 1 min). The initial intracellular concentration (C_i) was calculated by dividing the y-intercept of the linear portion of efflux time course by the cullular volume.

Furthermore, we obtained the uptake and efflux clearances (PS_{inf} and PS_{eff}, respectively), which was calculated as v/c after converting the v value to that per gram of liver, assuming that 1 mg protein contains 1.1×10^6 cells and 1 g liver contains 1.3×10^8 cells (Yamazaki *et al.*, 1992). Hence, the PS_{inf} or PS_{eff} value per gram of liver is expressed as follows;

$$\text{PS (mL/min/g liver)} = \frac{v\ (\text{nmol/min/mg protein})(1.3\times10^8)(\text{cells/g liver})}{c\ (\text{nmol/mL})(1.1\times10^6)(\text{cells/mg protein})} \quad (2)$$

where v and c represent the initial rate and ANS concentration in the space from which the flux occurs, respectively.

We also calculated the efflux clearance ($PS_{eff,u}$) for unbound ANS, using the binding parameters of ANS to cytosol determined with the equilibrium dialysis method ($n_{1(P)}$ = 8.22 μM, Kd_1 = 2.43 μM, $n_{2(P)}$ = 578 μM, Kd_2 = 259 μM) (Chung *et al.*, 1990b). The $PS_{eff,u}$ value was obtained by dividing the PS_{eff} value by the unbound fraction of ANS in hepatocytes. The relationship between the v_{eff} value and the unbound intracellular ANS concentration ($C_{i,u}$) was expressed as a Eadie-Hofstee plot, indicating that its efflux transport system is made up of a saturable and a nonsaturable components. Therefore, we fitted the efflux data to the following equation;

$$v_{eff} = \frac{V_{max}[S]}{K_{max}+[S]} + P_m[C_{i,u}] \quad (3)$$

where P_m represent the nonsaturable diffusion clearance (μL/min/mg protein).

RESULTS

Uptake of ANS by isolated hepatocytes

Fig. 1 shows the time courses of ANS uptake by isolated hepatocytes at its various concentrations (5-70 μM). The process of ANS uptake is linear within 60 sec for each concentration, and the initial uptake rate (v_{inf}) for each ANS concentration was calculated from the initial slope by a linear regression. When the uptake rates were plotted versus the intial ANS concentration, a hyperbolic curve demonstrating a saturation of ANS uptake process was obtained (Fig. 2A). A linear transformation of this curve (Eadie-Hofstee plot) yields a straight line (Fig. 2B).

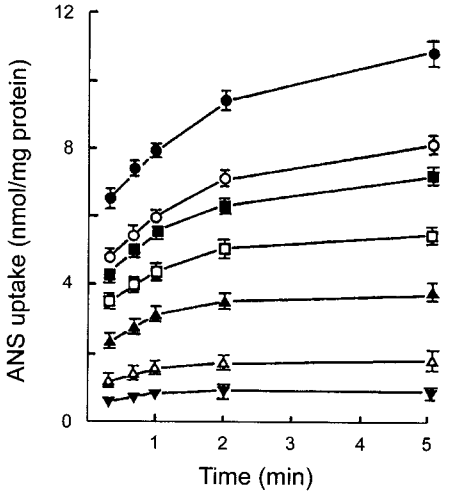

Fig. 1. Time course of uptake of ANS into isolated hepatocytes. Hepatocytes (1.7×10^6 cells/mL) were preincubated for 5 min at 37°C prior to the addition of ANS. The initial concentrations were ranged from 5 μM to 70 μM. Each point represents the mean±S.E. of three experiments. Keys: (▼), 5 μM; (△), 10 μM; (▲) 20 μM; (□), 30 μM; (■), 40 μM; (○), 50 μM; (●) 70 μM.

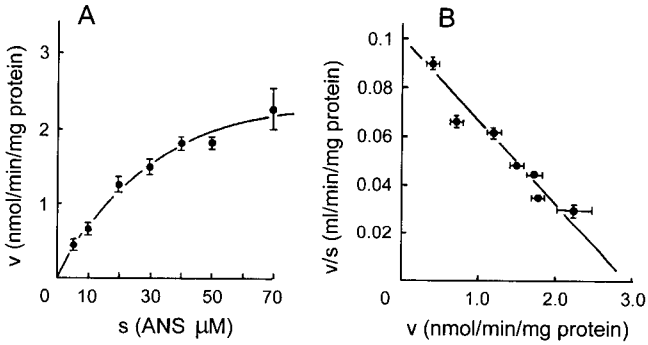

Fig. 2. Kinetics of ANS uptake into isolated rat hepatoyctes. (A) Plot of initial uptake rate vs. ANS concentration. (B) Eadie-Hofstee plot of ANS uptake into isolated hepatocytes. Initial uptake rate was measured within 1 minute of incubation, and was calculated by linear regression. Each point represents the mean±S.E. of three experiments.

The maximal uptake velocity (V_{max}) and the Michaelis-Menten constant (K_m) were calculated to be 2.90±0.10 (mmol/min/mg protein), and 29.1±3.20 μM, respectively. Under the pseudo steady state condition (at 5 min), the intracellular concentrations of ANS at initial medium concentrations of 5, 30 and 70 μM were higher than that in the incubation medium by factors of 125, 96, 65, respectively.

Efflux of ANS by isolated hepatocytes

The time courses of ANS efflux from isolated hepatocytes were depicted in Fig. 3. The amounts of ANS remaining in hepatocytes decayed exponentially within 2 min. The decay slope of ANS amount in hepatocytes representing its efflux rate constant was little decreased with intracellular

ANS concentration increased. Indeed, the efflux process for total ANS was independent on intracellular concentration (C_i) assessed from the y-intercept of the time course of ANS efflux (Fig. 4A). When the efflux clearance ($PS_{eff,u}$) for unbound ANS were plotted against the intracellular unbound concentration of ANS ($C_{i,u}$), the $PS_{eff,u}$ value was decreased with the $C_{i,u}$ values increased (Fig. 4B). The relationship between the v_{eff} and $C_{i,u}$ values was expressed as the Eadie-Hofstee plot (Fig. 5). The Eadie-Hofstee plot bent toward the horizontal axis as the v_{eff} value increased, indicating that ANS efflux system might be made up of two components, that is, a saturable and a nonsaturable components. The kinetic parameters calculated by fitting data to Eq. 4 were K_m = 10.0 μM and V_{max} = 0.54 mmol/min/mg protein for the saturable component, and P_m = 8.5 μL/min/mg protein for the nonsaturable component.

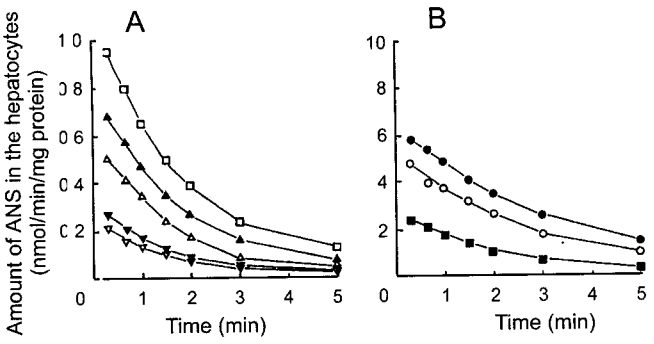

Fig. 3. Initial time course of ANS efflux from isolated hepatocytes. ANS at various concentrations (4-150 μM) was preloaded into hepatocytes (7×10^6 cells/mL) in 3 min at 37°C. After 3 min preloading, 2.5 mL of ANS preloaded cells were resuspended into 2.5 mL of 6% (w/v) BSA buffer. (A) and (B) represent the low and high ANS concentration in the medium, respectively. Each point represents the mean of two different preparations. Keys: (▽), 4 μM; (▼), 6 μM; (△), 10 μM; (▲), 15 μM; (□) 20 μM; (■) 50 μM; (○), 100 μM; (●) 150 μM.

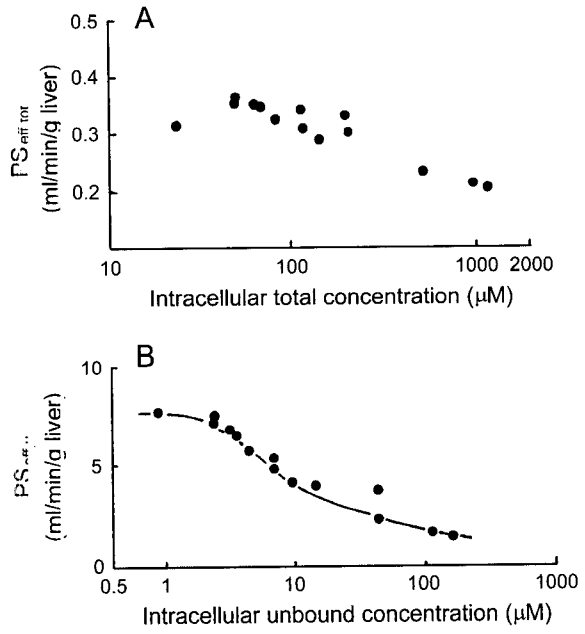

Fig. 4. Kinetics of ANS efflux from isolated rat hepatocytes. (A) Plot of efflux clearance for total ANS (PS_{eff}) vs. ANS intracellular concentration. (B) Plot of efflux clearance for unbound intracellular concentration of ANS vs. intracellular unbound ANS concentration. Efflux clearance was calculated by dividing the initial efflux rate by the intracellular concentration. The initial concentration of ANS was estimated from dividing the extrapolation of efflux time course by the intracellular volume. The unbound intracellular ANS concentration was calculated, based on the binding parameters determined with the cytosol (Chung *et al.*, 1990b). Each point represents the mean of two different experiments.

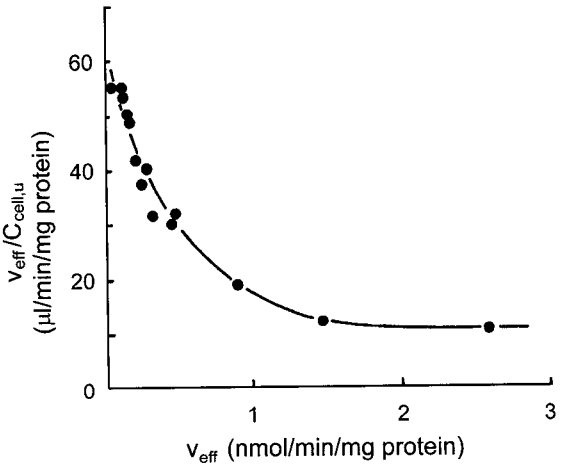

Fig. 5. Eadie-Hofstee plot of ANS efflux process from isolated hepatocytes. $C_{i,u}$ represents the unbound intracellular concentration of ANS determined with its binding parameters to cytosol (Chung *et al.*, 1990b). Each point represents the mean of two different experiments.

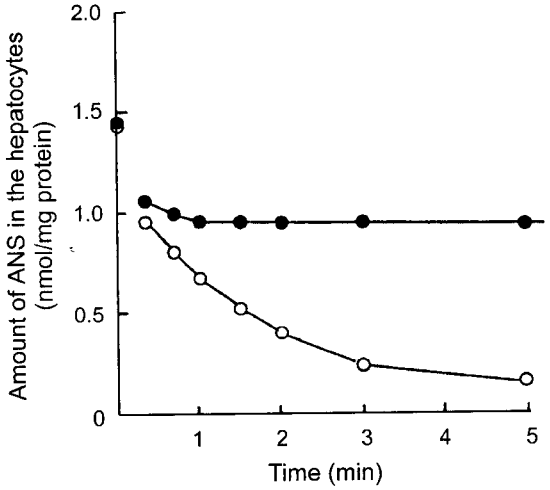

Fig. 6. Temperature-dependence of the efflux from isolated hepatocytes. ANS (20 μM) was preloaded into the hepatocytes (7×10^6 cells/mL) in 3 min at 37°C. ANS proloaded cells (initial intracellular concentration: 100 μM) were resuspended into 4°C (●) and 37°C (○) 3% (w/v) BSA buffers. Each point represents the mean of two different experiments.

Temperature-dependency of ANS efflux from isolated hepatocytes

Temperature-dependency of ANS efflux from isolated hepatocytes is depicted in Fig. 6. ANS efflux was completely inhibited at low temperature (4°C). The y-intercepts of the time courses of ANS efflux at 37°C and 4°C were similar, suggesting that the disappearance of ANS from the hepatocytes might reflect the efflux process of intracellular ANS. On the other hand, the amount of ANS in the hepatocytes at time zero (prior to the dilution) was somewhat larger than the y-intercept of this time course. This could be explained by a rapid dissociation of ANS adsorbed to the cell surface.

DISCUSSION

Comparing the kinetics of ANS uptake and efflux processes by rat hepatocytes, we have characterized the bidirectional hepatic tansport of ANS in the present study. The uptake process of ANS was saturable with a K_m of 29.1 μM, V_{max} of 2.9 mmol/min/mg protein, being compatible with Sugiyama's study (Sugiyama *et al.*, 1983), in which they determined the ANS taken up by the hepatocytes with assessement of the fluorescence-change. While the efflux process for total ANS were little changed with ANS increased. We then corrected the efflux process for the intracellular unbound concentration of ANS, based on the binding parameters of cytosol (Chung *et al.*, 1990b). The $PS_{eff,u}$ value was decreased in a unbound intracellular concentration dependent manner (Fig. 4B). As shown in the Eadie-Hofstee plot (Fig. 5), the efflux process of ANS revealed a saturable and a nonsaturable transport components. The kinetic parameters calculated of ANS efflux process based on Eq. 4 were K_m = 10.0 μM, and V_{max} = 0.54 nmol/min/mg protein for the saturable component. Comparing the ratio of V_{max} to K_m value, which reflects the transport ability, the V_{max}/K_m value for uptake process was approximately two times higher than that for efflux process. This results suggested that ANS might be energy-dependently taken up by hepatocytes. Indeed, the ANS influx process was substantially inhibited by the ATP suppression in hepatocytes. Taken together, the two criteria on active tranport were satisfied in the bidirectional hepatic transport of ANS; one is that uptake of ANS can occur against a chemical potential, the other is that metabolic energy is required for ANS transport. On the other hand, Sugiyama's study (Sugiyama *et al.*, 1983) with isolated hepatocytes demonstrated that the initial uptake rate of ANS was unaffected by metabolic inhibitors, such as ouabain (1 mM), rotenone (10 μM). It is likely that in their study, concentration of metabolic inhibitor and its exposure time were not enough to suppress the ATP level in hepatocytes. However, unfortunately, studies on the inhibition of the ANS uptake by concentration of metabolic inhibitor low enough for relevant kinetical data have not be performed yet.

Based on Eq. 2, we calculated the PS_{inf} value in a whole liver. The PS_{inf} value determined with isolated hepatocytes is 13.7 mL/min/g liver. While, the PS_{inf} value assessed from the analysis of the plasma disappearance curve of ANS after iv. administration were 10 mL/min/g liver, revealing the good coincidence with that in the present study (Chung *et al.*, 1990b). These suggested that the PS_{inf} value estimated with isolated hepatocytes might reflect the permeability in the *in vivo*, and this method might be suitable to the characterization of the bidirectional transport of ANS. Furthermore, the PS_{eff} value determined with isolated hepatocytes was also comparable with that assessed in the *in vivo* experiment. These suggest that the hepatic transport of ANS assessed with isolated hepatocytes might be reliable and accurate.

The isolated hepatocytes model does not allow for discrimination between releases of ANS via the bile canalicular and the sinusiodal membranes. However, on the basis of morphometric studies, the total sinusoidal membrane surface area would be expected to approximately seven times larger than that of canalicular membrane. Since there is also little flux of ANS via biliary excretion process (less than 2% of ANS taken up by hepatocytes) (Chung *et al.*, 1990b), it is likely that the present observation on the efflux of ANS from isolated hepatocytes related to release via the sinusoidal membrane.

Recently, we clarified ANS metabolism in the hepatocytes, using isolated microsome (Chung *et al.*, 1998). In the previous studies, microsomal mono-oxygenase system was found to be mainly responsible for the hepatic metabolism of ANS. The intrinsic clearance of metabolism for total ANS (CL_{int}) was calculated to be 0.15 mL/min/g liver, based on the metabolic parameters (Chung *et al.*, 1990b). The CL_{int} value was approximately one-third fold lower than the PS_{eff} value, suggesting that the metabolism of ANS taken up by hepatocytes might be minimal within 5 min. Indeed, the total recovery of ANS amounts in the medium and hepatocytes revealed more than 96% for each ANS concentration. Therefore, the underestimation of the uni-directional efflux rate from hepatocytes caused by ANS intracellular metabolism might be minimal in the present study.

In conclusion, ANS is actively taken up into hepatocytes via a carrier mediated transport system. The influx process of ANS revealed the requirement of the energy for transport.

CONCLUSION

The uptake process of ANS was saturable, with a K_m of 29.1±3.2 μM and V_{max} of 2.9±0.1 nmol/min/mg protein. The

V_{max} and K_m values were 0.54 nmol/min/mg protein, and 10.0 μM, respectively in the efflux process for the unbound intracellular ANS concentration. Based on the comparison of the ratios of V_{max} to K_m (V_{max}/K_m) corresponding to the transport clearance, the influx clearance was two times higher than the efflux clearance. These results indicated that the hepatic uptake of ANS is actively taken up into hepatocytes via the carrier mediated transport system.

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