Quantitative Evaluation of Brain Distribution and Blood-Brain Barrier Efflux Transport of Probenecid in Rats by Microdialysis: Possible Involvement of the Monocarboxylic Acid Transport System

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ABSTRACT

This study was performed to evaluate quantitatively the brain distribution and the efflux transport across the blood-brain barrier of probenecid, using in vivo microdialysis and in situ brain perfusion techniques. The brain interstitial fluid (ISF)-to-plasma cerebrospinal fluid (CSF)-to-plasma and brain tissue-to-plasma unbound concentration ratios of probenecid at steady state were less than unity, which suggests restricted distribution in the brain. An uphill concentration gradient from ISF to plasma and a downhill concentration gradient from CSF to ISF were observed. Kinetic analysis revealed that the efflux clearance from brain ISF to plasma (0.0373 ml/min/g brain) was significantly greater than the influx clearance from plasma to brain (0.00733 ml/min/g brain). The ratio of the ISF concentration (C_{ISF}) to the plasma unbound concentration (C_{p,f}) of probenecid was increased 2- to 3-fold by salicylate (3.7 mM) and benzoate (3.6 mM), which are accepted as substrates of the monocarboxylic acid transport system, compared with the same ratio for the control. In addition, the ratio C_{ISF}/C_{p,f} was increased by treatment with N-ethylmaleimide, a sulfhydryl-modifying agent, whereas p-aminohippuric acid and choline did not produce increasing effects on C_{ISF}/C_{p,f}. These data suggest that the restricted distribution of probenecid in the brain may be ascribed to efficient efflux from the brain ISF, which may be regulated by the monocarboxylic acid transport system at a relatively high ISF concentration.

Clarification of the brain distribution of centrally acting neuropharmaceuticals after systemic administration is important to better understanding their pharmacological effects in the central nervous system. As a rule, the drug concentration in the brain has been known to be regulated by several factors, such as plasma protein binding, transport property between blood circulation and brain ISF across the BBB, intracellular-to-ISF exchange, diffusion between the ISF space and the CSF pool and transport between blood circulation and CSF via the choroid plexus (Collins and Dedrick, 1983; Morrison et al., 1991). In particular, it has generally been accepted that passage from blood circulation to the brain via a lipid-mediated and specific transport system at the BBB is one of the dominant factors influencing the brain concentration of a drug.

On the other hand, Conford et al. (1985) have pointed out that efficient efflux of valproic acid, an antiepileptic drug, from the brain may be responsible for its restricted distribution in the brain. Furthermore, because it has been found that p-glycoprotein is functioning as a drug efflux pump at the BBB (Cordon-Cardo et al., 1989; Tsuji et al., 1992), the importance of efflux transport across the BBB has been increasingly recognized. For example, recent reports have provided in vivo evidence that the anti-AIDS drug zidovudine (Wong et al., 1993; Dykstra et al., 1993) and valproic acid (Adkison et al., 1994) are recognized by a probenecid-sensitive efflux system at the BBB. Recently, we reported that the

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ABBREVIATIONS: A_{tot}, total amount taken up by the brain; BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; BCSFB, blood-cerebrospinal fluid barrier; BSA, bovine serum albumin; C_{infusion}, infusion dose given into the brain interstitial fluid; C_{int}, concentration in interstitial fluid; C_{p,f}, unbound concentration in plasma; C_{perfusate}, concentration in perfusate; C_{CSF}, cerebrospinal fluid clearance; C_{inf}, influx clearance from plasma to brain; C_{eff}, efflux clearance from brain to plasma; CSF, cerebrospinal fluid; f_{p,u}, unbound fraction in plasma; ISF, interstitial fluid; K_{eff}, efflux rate constant from brain to plasma; K_{i}, inhibition constant; K_{m}, Michaelis constant; K_{R}, Krebs-Ringer phosphate buffer; MeAIB, methylaminoisobutyric acid; MCT, monocarboxylic acid transport; NEM, N-ethylmaleimide; NMN, N’-methyleneicotinamide; PA_{vivo}, in vivo permeability rate constant of the microdialysis probe; PA_{in vitro}, in vitro permeability rate constant of the microdialysis probe; R_{vivo}, in vivo recovery of the microdialysis probe; R_{in vitro}, in vitro recovery of the microdialysis probe; T_{1/2}, elimination half life in β-phase; V_{d}, volume of distribution in the brain.
restricted distribution of baclofen, an antispastic drug, may also be due to efficient efflux from the brain, possibly by a probenecid-sensitive system at the BBB (Deguchi et al., 1995). These results suggest that administration of probenecid as an adjunct to drug treatment might be useful for improving the restricted brain distribution of a drug that is recognized by the probenecid-sensitive efflux system. However, there are few reports about the brain ISF distribution and the BBB efflux transport of probenecid itself, factors that would be important in the probenecid-drug interaction. Probenecid possesses a carboxyl residue (pKₐ = 3.4) in its chemical structure (Weiner, 1990) and has been a valuable tool in studies characterizing the transport mechanism of organic anions. It has been demonstrated in a study using renal cortical slices that probenecid itself can be transported via the common organic anion transport system as PAH with a Kₐ value of 40 μM (Sheikhand Maxild, 1978). In contrast, however, it has been reported that probenecid inhibits competitively the transport of NMN, a classical substrate of the organic cation transporter, in renal brush-border membrane vesicles (Hsyu et al., 1988). In addition, probenecid has been reported to block the uptake of L-lactate into mouse peritoneal macrophages via a probenecid-inhibitable MCT system, which is distinct from the anion exchange system (Loike et al., 1993). These findings would lead to the hypothesis that probenecid is recognized not only by the organic anion transport system, as is PAH, but also by several other transport systems, at the BBB.

The purpose of this study, therefore, was to assess quantitatively the brain distribution and the efflux transport of probenecid at the BBB, using the in vivo microdialysis technique and several other in vivo techniques. In this report, the authors will also report results that suggest that the MCT system plays an important role in the efflux transport of probenecid across the BBB.

Materials and Methods

Materials. The following drugs and chemicals were used in this study: probenecid (MW 285.4), sodium salicylate (MW 160.1), choline chloride (MW 139.6) (Wako Pure Chemical Industries Ltd., Tokyo, Japan), NMN (MW 136.2) and PAH sodium salt (MW 216.2) (Sigma Chemical Co., St. Louis, MO), sodium benzoate (Kanto Chemical Co., Inc., Tokyo, Japan), [¹⁴C]sucrose (MW 342.3, specific activity 17.6 GBq/mmoll) and α-[¹⁴C]MeAIB (MW 117.1, specific activity 2.1 GBq/mmoll) (Dupont New England Nuclear, Life Science Products, Boston, MA). All other chemicals were of analytical grade and were used without further purification.

Animals. Adult male Wistar rats weighing 250 to 300 g were purchased from Japan SLC (Shizuoka, Japan); they were housed, three or four per cage, in a laboratory with free access to food and water and were maintained on a 12-hr dark/12-hr light cycle in a room with controlled temperature (24°C ± 2°C) and humidity (55% ± 5%).

Surgical procedures for brain microdialysis. Surgical procedures for implantation of the horizontal-type microdialysis probe have been described by Deguchi et al. (1991) and Terasaki et al. (1992). Briefly, the microdialysis probe was made from Cuprophan hollow-fiber membrane (ID 0.2 mm, wall thickness 11 μm; MW cutoff 12,500; RENAK-E, RE-10M, Kawasaki Chemical Industries Ltd., Tokyo, Japan) and stainless steel tubing (OD 0.2 mm; MT Giken, Tokyo, Japan). The length of the diffusible part of the probe was 8 mm.

Rats were anesthetized with ketamine (235 mg/kg i.m.) and placed in a stereotaxic frame (SR-6, Narishige Scientific Instrument Lab., Tokyo, Japan). The microdialysis probe was horizontally implanted in the hippocampus region (3.4 mm posterior to the bregma and 3.5 mm below the dura) while KRP buffer (120 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 0.9 mM NaH₂PO₄, 1.4 mM Na₂HPO₄, pH 7.4) was perfused through the probe at a constant flow rate of 5 μl/min. The probe-bearing rat was allowed free access to food and water for 48 hr.

Intravenous administration study. The probe-bearing rat was anesthetized with ketamine (118 mg/kg), and its left femoral artery and right femoral vein were cannulated with polyethylene tubing (SP-31, Natsume Seiskakuso Ltd., Tokyo, Japan) filled with heparin-saline solution (100 U/ml), for the blood sampling and the i.v. infusion, respectively. One side of the microdialysis probe was connected to polyethylene tubing (SP-10) and perfused with KRP buffer, at a constant flow rate of 5.0 μl/min, by means of a precision infusion pump (Model 22, Harvard Apparatus, South Natick, MA). After a 30-min stabilization period, probenecid was infused i.v. via a femoral vein at a constant rate of 75 mg/kg/hr. Infusion was terminated at 210 min. Dialysate samples were collected at 10-min or 20-min intervals for 440 min, and blood was withdrawn through the cannula at appropriate times.

To obtain the steady-state concentrations of probenecid in plasma and brain, a bolus dose of probenecid (99 mg/kg) was administered i.v. to rats, followed by an i.v. infusion at a constant rate of 26.6 mg/kg/hr. Dialysate was collected at 10-min or 20-min intervals, and blood was withdrawn via a femoral vein at appropriate times. After 210 min, CSF (100 μl) was removed by cisternal puncture (Chow and Levy, 1981) just before decapitation, and a blood sample (5 ml) was withdrawn through the cannula. Then the rats were decapitated, and the brain tissue was removed, rinsed with cold saline, blotted and weighed.

A pilot study showed slow disappearance of salicylate from plasma (T½ was approximately 30 hr). Thus salicylate was administered i.v. at a bolus dose of 100 mg/kg; thereafter, dialysate and plasma were collected at 150 to 210 min. Samples of brain tissue and CSF were collected at 210 min.

Intra-ISF infusion. Drugs were administered into the brain ISF by perfusing the microdialysis probe with the dialysis solution containing probenecid (2 mM or 120 mM, pH 7.4, 300 mM) or [¹⁴C]MeAIB (308.3 kBq/ml) with unlabeled MeAIB (100 μg/ml). After a 30-min perfusion, the solution was switched to KRP, and dialysate samples were collected at 10-min intervals for 120 min. In vitro dialysis in an agar gel plate (0.5%) was carried out in a similar manner. [¹⁴C]MeAIB was used as a diffusion marker in brain parenchymal tissue in the present study.

Measurement of the CLₘ across the BBB. The CLₘ values of probenecid and salicylate were determined by the i.v. administration method (Blasberg et al., 1983) or the in situ brain perfusion technique (Takasato et al., 1984; Triguero et al., 1990).

In the i.v. administration method, probenecid was administered i.v. to ketamine-anesthetized rats at a dose of 75 mg/kg. Blood and brain tissue were sampled at 1, 3, and 5 min for the integration plot analysis.

In the in situ brain perfusion, rats were anesthetized with ketamine (235 mg/kg), and the occipital and superior thyroid arteries were cut off by electrocoagulation (Janus Bipolar Coagulator, Keisei Medical Industrial Co., Tokyo, Japan). The right external carotid artery was catheterized with polyethylene tubing (SP-10), and the common carotid artery was closed just before the perfusion was started. The perfusate consisted of Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM d-glucose, 0.1% BSA, pH 7.4) containing antipyrine (5.3 mM), an availability marker and [¹⁴C]sucrose (6.6 kBq/ml), an intravascular marker of brain perfusion. The perfusate was bubbled for 5 min with O₂/CO₂ (95:5) before addition of the BSA (0.1%), probenecid (700 μM) and/or salicylate (1250 μM), and it was maintained at 37°C. Perfusion was carried out at a flow rate of 1.2
ml/min with an infusion pump (Model 22, Harvard Apparatus, South Natick, MA). For the longer perfusion times (> 3 min), blood was withdrawn from the femoral artery at approximately half of the perfusion flow rate. At 1, 3, 5 and 10 min, rats were decapitated, and the ipsilateral hemisphere to the perfusion side was removed, rinsed with cold saline, blotted and weighed.

**Measurement of CL_{srf}**. The CL_{srf} value of probenecid and salicylate was estimated from the concentrations in ISF, brain and plasma at steady state, and the CL_{srf} value (Details of this calculation appear in the "Data Analysis" section).

**Measurement of CL_{out}**. The CL_{out} of probenecid after i.v. administration was measured according to the method described by Ogawa et al. (1994). Briefly, rats were anesthetized with ketamine (235 mg/kg, i.m.); the heads were fixed in a stereotaxic apparatus, and a hole was made in the skull (0.5 mm posterior to and 2 mm to the left side of the bregma). A stainless steel needle (OD 0.45 mm, ID 0.1 mm, length 15 mm; EICOM, Kyoto, Japan) connected to a polyethylene tube (SP31) was inserted into the left lateral ventricle. Ten microliters of a solution containing probenecid (45.6 mM) dissolved in artificial CSF (122 mM NaCl, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 25 mM NaHCO3, 0.4 mM K2HPO4, 10 mM t-glucose, 10 mM HEPES, pH 7.3, equilibrated with 95% O2–5% CO2 gas at 37°C) was drawn at the time designated. This solution was injected onto an HPLC column. Dialysate samples were directly diluted with an appropriate volume of KRP, and a 30-μl aliquot was injected onto an HPLC column.

**Determination of the unbound fraction in plasma**. The plasma unbound fraction was measured by the ultrafiltration method described previously (Deguchi et al., 1995).

**Determination of R_{tini} values of the microdialysis probe**. The R_{tini} and PA_{tini} values of probenecid, salicylate and [14C]MeAIB were measured by the method described previously (Deguchi et al., 1991).

**Effect of NEM treatment on the ISF concentration of probenecid**. NEM has been reported to reduce the transport activity by irreversibly modifying sulfhydryl groups of the protein, such as the monocarboxylic acid transporter (Loike et al., 1993). The irreversible nature of the binding of NEM to protein would allow the decreased activity of the efflux transport to persist for a long time. Thus the rat hippocampal proteins were modified by perfusing the dialysis solution containing NEM (1 mM) through the microdialysis probe for 90 min at a constant flow rate of 5 μl/min. Then the probe was washed out by perfusing with KRP for 60 min, to remove the excess NEM remaining in the probe. Two hours later, probenecid was administered i.v. at a bolus dose of 75 mg/kg. Dialysate samples were collected at 20-min intervals for 180 min, and blood was withdrawn at the midpoint of each dialysate collection period. CSF was withdrawn 180 min later. These experimental designs would exclude the unexpected mutual interaction between probenecid and NEM, and the reversible interaction between transporters and NEM, by the addition of NEM after the i.v. bolus administration of probenecid.

**Inhibition studies**. Probenecid was administered i.v. to rats at doses of 75 mg/kg. The microdialysis probe was perfused with KRP for the first 60 min after the bolus injection of probenecid. For the next 60 min, the KRP was switched to the solution (pH 7.4, 256.8 mOsm) containing several compounds (inhibitors), such as salicylate (50 mM), benzoate (50 mM), PAH (50 mM), NMN (50 mM) and choline (50 mM), to infuse these compounds into the brain ISF.

In the case of i.v. administration of salicylate, the microdialysis probe was perfused with KRP for 180 min after i.v. bolus administration of salicylate (100 mg/kg). Then the KRP was switched to the isotonic solution (pH 7.4) containing several compounds (inhibitors), such as probenecid (30 mM, 300 mOsm), benzoate (50 mM, 256.8 mOsm) and NMN (50 mM, 256.8 mOsm). Dialysate was collected at 10-min intervals, and blood was withdrawn at the time designated.

**Effects of NEM and inhibitors on the initial brain uptake and intravascular space**. The initial brain uptake of probenecid in rats treated with NEM was measured by the i.v. administration method in a manner similar to that described above. In addition, the initial brain uptakes of probenecid and salicylate, and the sucrose space, in rats in which salicylate (50 mM) and probenecid (50 mM) were infused as inhibitors into the brain ISF for 30 min, were measured by means of *in situ* brain perfusion. The perfusates containing probenecid (700 μM), salicylate (1250 μM) and [14C]sucrose (6.6 kBq/ml) were prepared with Krebs-Henseleit buffer. After a 5-min perfusion, rats were decapitated and the ipsilateral hemispheres removed.

**Analytical procedure**. The concentrations of probenecid and salicylate in dialysate, plasma, CSF, and brain tissue were determined by an HPLC method based on the report of Galinsky et al. (1991). An aliquot of plasma sample was mixed with an equal volume of KRP buffer and a 4-fold volume of acetonitrile and was allowed to stand for 30 min at −20°C; then the sample was centrifuged at 10,000 rpm for 5 min. The supernatant was diluted with an appropriate volume of KRP. A 30-μl aliquot was injected onto an HPLC column.

Brain tissues were homogenized with a 2-fold volume of KRP buffer and a 4-fold volume of acetonitrile, in a glass homogenizer in an ice-cold bath, and were allowed to stand for 1 hr at −20°C; then each sample was centrifuged twice at 10,000 rpm for 5 min for proteineinization. The supernatant was filtered by the hydrophilic polytetrafluoroethylene membrane (Samprep-LCR-4T/LH; pore size 0.5 μm; Nihon Millipore Ltd., Tokyo, Japan), and a 30-μl aliquot was injected onto an HPLC column. Dialysate and CSF samples were directly diluted with an appropriate volume of KRP, and a 30-μl aliquot was injected onto an HPLC column.

The HPLC system consisted of a pump (880-PU, Japan Spectroscopic Co. (Jasco), Tokyo, Japan), a UV detector (870-UV, Jasco) and an integrator (Chromatocorder 12, System Instruments, Co. Ltd., Tokyo, Japan). The HPLC analytical column was a Finepack SIL C_{18}S ODS (4.6 mm I.D. × 25 cm length, 5-μm particle size, Jasco), and the guard column was a μ-Bondapack C_{18}, Guard-Pak Insert (Waters, Milford, MA).

The analytical conditions for probenecid were as follows. The flow rate was 1.0 ml/min, and the column eluate was monitored at a UV wavelength of 254 nm. The mobile phase was 0.01 M KH_{2}PO_{4}-acetonitrile = 780:220 (v:v), except for samples obtained in the inhibition study. When benzoate and NMN were used, the mobile phase was 0.01 M KH_{2}PO_{4}-acetonitrile = 810:190 (v:v). When PAH was used as an inhibitor, the mobile phase was 0.01 M KH_{2}PO_{4}-acetonitrile = 820:180 (v:v). The peak area was used for quantification. The concentration was determined from the calibration curve prepared by the same procedure as that for the respective sample. The detection limit was 0.35 μM.

Salicylate was detected under the following analytical conditions. UV wavelength was 300 nm, and the mobile phase was 0.01 M KH_{2}PO_{4}-acetonitrile = 950:50 (v:v). When benzoate was used as an inhibitor, the mobile phase was 0.01 M KH_{2}PO_{4}-acetonitrile = 980:20 (v:v). The detection limit was 0.18 μM.

**Data analysis**. CL_{srf} was estimated by the reference method of equation (1) (Deguchi et al., 1991; Terasaki et al., 1992).

\[
CL_{srf} = \frac{C_d}{1 - \exp(-R_{tini} PA_{tini}/F)}
\]

where C_d is the concentration in dialysate and F is the dialysis flow rate. R_{tini} is the effective dialysis coefficient of the reference compound, antipyrine; we used 0.389, the value reported previously by Terasaki et al. (1992). This equation was previously derived from clearance theory in pharmacokinetics, on the basis of the assumption that the microdialysis system is in the steady-state condition (Deguchi et al., 1991). In addition, our previous study (Deguchi et al., 1995) suggested that this method would be applicable for estimating the ISF concentration after bolus administration of a drug, without a significant error of estimation. Accordingly, equation (1) was used to...
estimate the ISF concentration throughout the present study. However, the estimated concentration should be recognized as a good approximation to a real value.

$$CL_{in} = \frac{A_{m} + C_{perfusate} - V_{isf}}{fT}$$

where \(A_{m}\) and \(C_{perfusate}\) are the total amount per gram of brain and the concentration in perfusate, respectively. \(T\) is the perfusion time. \(V_{isf}\) is the intravascular space determined by \(^{14}\text{C}\) sucrose. The availability of perfusion, \(f\), was obtained from the relationship

$$f = V_{ap}/0.91$$

where \(V_{ap}\) is the volume of distribution, defined as \(A_{m}/C_{perfusate}\) of antipyrine, after a 5-min perfusion. The value 0.91 represents the water space allowing antipyrine to equilibrate in the brain (Sakurada et al., 1978).

\(CL_{in}\) was also estimated by integration plot analysis (Blasberg et al., 1983), on the basis of the data obtained by the i.v. administration method.

$$A_{m} \times C_{psf}(\tau) d \tau / C_{psf} + V_{isf}$$

where \(C_{p,t}\) and \(C_{p,s}\) are the total and the unbound concentrations in plasma at time \(t\), respectively. \(V_{isf}\) is the functional volume of distribution that rapidly exchanges with plasma.

\(CL_{out}\) was calculated according to equations (5) to (7) on the basis of the method reported previously (Deguchi et al., 1995).

$$k_{dff} = CL_{in} \times f_{p} / X_{m,ss}$$

The term \(k_{dff}\) is the efflux rate constant from brain to plasma derived from the steady-state condition of the two-compartment single-membrane model. \(C_{psf}\) is the steady-state concentration in plasma. \(X_{m,ss}\) is the steady-state amount taken up by the brain, which was estimated by subtracting the amount remaining in the vascular space \((V_{isf} \times C_{psf})\) from the total amount in the brain at the steady state \((A_{m,ss})\). The volume of distribution in the brain \(V_{isf}\) was defined previously (Deguchi et al., 1995) and represents the partition of brain parenchymal cells to interstitial fluid. \(CL_{out}\) is a parameter that reflects not only the clearance via the BBB but also, in part, the clearance from the CSF pool to plasma.

Statistical analysis. All data are presented as mean ± S.E., except as otherwise noted. Student’s \(t\) test was used to compare individual means. The effect of inhibitors on the \(C_{eff}/C_{p,t}\) ratio for probenecid and salicylate was statistically tested by one-way analysis of variance with multiple comparison (Duncan’s New Multiple-Range test), to examine differences between the control and inhibitor-treated groups.

**Results**

**In vitro recovery of microdialysis probe.** The \(R_{\text{vitro}}\) and \(PA_{\text{vitro}}\) values of probenecid, salicylate and \(^{14}\text{C}\)MeAIB are listed in table 1. The \(PA_{\text{vitro}}\) values estimated here corresponded well with those predicted from the linear relationship between MW\(^{0.5}\) and the reciprocal of \(PA_{\text{vitro}}\) reported by Deguchi et al. (1991). The \(PA_{\text{vitro}}\) values of benzoate, PAH, NMN and choline were predicted from this line.

**In vitro and in vivo plasma protein binding.** The \(f_{p}\) value of probenecid, determined by the in vitro ultrafiltration method, was 0.461 ± 0.014 (\(n = 3\)) at the total plasma concentration of 1021 ± 54 \(\mu\)M (\(n = 3\)). The \(f_{p}\) value of salicylate was 0.413 ± 0.025 (\(n = 3\)).

**Infusion study of probenecid.** Figure 1 shows profiles for the plasma and hippocampal ISF concentrations of probenecid vs. time after a constant i.v. infusion of probenecid.

The level of unbound concentration in plasma, estimated using the \(f_{p}\) value, is also shown. The plasma concentration of probenecid increased with time, reaching a peak at the termination of the infusion, and then declined very slowly. The probenecid concentration in the ISF changed in parallel with that in plasma, and it was never greater than the unbound concentration in plasma. The levels of ISF concentration were approximately 5-fold lower than those of the unbound concentration in plasma over the period of the experiment.

**Steady-state concentrations in the brain.** The ISF-to-plasma, CSF-to-plasma and brain tissue-to-plasma unbound concentration ratios of probenecid at steady state were 0.199 ± 0.017 (\(n = 3\)), 0.629 ± 0.041 (\(n = 3\)) and 0.482 ± 0.015 (\(n = 3\)), respectively, when the unbound concentration in plasma was 348.5 ± 14.7 \(\mu\)M (\(n = 3\)). As depicted in figure 2A, we observed an uphill concentration gradient from ISF toward plasma and a downhill concentration gradient from the CSF pool toward the ISF.

In the case of salicylate, the ISF-, CSF-, and brain tissue-to-unbound concentration ratios at steady state were 0.126 ± 0.024 (\(n = 3\)), 0.462 ± 0.028 (\(n = 3\)) and 0.352 ± 0.033 (\(n = 3\)), respectively, when the unbound concentration in plasma was 878 ± 54 \(\mu\)M (\(n = 3\)).

![Fig. 1. The concentrations of probenecid in plasma (●) and hippocampal ISF (○) after an i.v. infusion (75 mg/kg/hr) to rats. Infusion was terminated at 210 min. Each point represents the mean ± S.E. of 3 to 4 determinations. The solid line shows the level of unbound probenecid concentration in plasma.](image-url)
Estimation of CL_{in}, CL_{out} and V_d. Figure 3A shows the result of the initial brain uptake of probenecid obtained by the in situ brain perfusion technique. Uptake of probenecid by the brain was linear over the perfusion time of 1 to 10 min. The C_{isf}/C_{perfs} ratio, calculated according to equation (8) from the results of the 5-min perfusion, was 0.0196 ± 0.0033. The value was less than 10% of the C_{isf}/C_{perfs} value at steady state (0.199), which suggests that the efflux (back-flux) makes a minor contribution to the CL_{in} value during the 5-min perfusion. The estimated CL_{in} value is given in table 2.

\[
C_{\text{isf}} = \frac{A_{\text{m}} - V_d C_{\text{perfs}}}{fV_d}
\]

Parameters are defined in “Materials and Methods.”

Further, as shown in figure 3B, the CL_{in} and V_i values were estimated by integration plot analysis (equation 4) from the data of initial brain uptake after an i.v. bolus administration of probenecid. The results were 0.00794 ± 0.00188 ml/min/g brain (mean ± S.D., n = 9) for CL_{in} and 0.0196 ± 0.0033 ml/g brain (mean ± S.D., n = 9) for V_i, which values were consistent with those obtained by the in situ brain perfusion technique.

The values of CL_{out}, k_{eff} and V_d are listed in table 2. The CL_{out} value was 5-fold greater than the CL_{in} value. The V_d value for probenecid was approximately 2 ml/g brain, which suggests that probenecid is taken up by the brain parenchymal cells and binds to the organelle and cell or that probenecid is concentrated into the brain parenchymal cells by the synaptosomal membrane.

The BBB transport and brain distribution parameters of salicylate were also determined (table 2). The CL_{out} value was significantly 8-fold greater than the CL_{in} value.

**Disappearance after intra-ISF administration.** Figure 4 shows the disappearance curves of probenecid from the

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**TABLE 2**

Parameters for the plasma-brain transport and distribution in the brain of probenecid and salicylate in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Probenecid</th>
<th>Salicylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL_{in} (ml/min/g brain)</td>
<td>0.0073 ± 0.00218</td>
<td>0.00864 ± 0.00168</td>
</tr>
<tr>
<td>CL_{out} (ml/min/g brain)</td>
<td>0.0373 ± 0.0052*</td>
<td>0.0736 ± 0.0210*</td>
</tr>
<tr>
<td>k_{eff} (min⁻¹)</td>
<td>0.0179 ± 0.0010</td>
<td>0.0324 ± 0.0066</td>
</tr>
<tr>
<td>V_i (ml/g brain)</td>
<td>2.10 ± 0.39</td>
<td>2.30 ± 0.71</td>
</tr>
<tr>
<td>CL_{isf} (ml/min/g brain)</td>
<td>0.0221 ± 0.0034</td>
<td>—</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. of 3 to 4 determinations.

* Significantly different from CL_{in} (P < .05).
brain ISF after the intra-ISF infusion of probenecid. Both the low-dose and the high-dose groups initially declined at a relatively rapid rate and then decreased with time at a relatively slow rate. The lines were superimposed on each other. The half-lives of the disappearance rate from ISF around the microdialysis membrane (≥ 25 min) were 13.8 ± 1.9 min for the low-dose group and 15.2 ± 0.5 min for the high-dose group. No significant differences were observed between them, which suggests that there is no dose dependence in the range of 2 to 120 mM.

On the other hand, the ISF concentration of [14C]MeAIB, which was used as a diffusion marker in brain parenchymal tissue, decreased slowly with time, except for an initial decline. Moreover, as shown in figure 4B, the disappearance rates of both probenecid and [14C]MeAIB in the gel matrix around the microdialysis probe were slow, and they were superimposed on that of [14C]MeAIB in the brain. The half-life of the disappearance rate (≥ 25 min) was 63.3 ± 7.4 min for [14C]MeAIB (gel matrix), 49.2 ± 5.5 min for probenecid (gel matrix) and 48.1 ± 5.5 min for [14C]MeAIB (in vivo). There are no statistically significant differences between [14C]MeAIB (gel matrix) and probenecid (gel matrix), and also between [14C]MeAIB (gel matrix) and [14C]MeAIB (in vivo).

Effect of NEM treatment on the C_{isf/C_{p,f}} ratio. The effect of NEM treatment on the C_{isf/C_{p,f}} ratio for probenecid is shown in figure 5A. The C_{isf/C_{p,f}} value for probenecid after NEM treatment (0.296 ± 0.010, n = 5) increased significantly compared with that of the control (0.140 ± 0.001, n = 6), although the level of unbound concentration in plasma remained unchanged. On the other hand, initial brain uptake of probenecid in the hippocampus and the right hemisphere (A_{m}/C_{p,f}) were not changed by the NEM treatment (fig. 5B), which suggests that NEM treatment did not bring about significant enhancement of C_{isf/C_{p,f}}.

Effect of several compounds (inhibitors) on the C_{isf/C_{p,f}} ratio for probenecid and salicylate. To reveal the relationship between the efflux transport system of probenecid and salicylate and the endogenous transport system at the BBB, we conducted inhibition studies using several compounds (inhibitors) that are known to be accepted by the endogenous transport system. Table 3 lists the effects of inhibitors on the C_{isf/C_{p,f}} value for probenecid. The ISF concentrations of inhibitors, estimated by the PA_{vivo}, values (C_{isf/C_{p,f}}^\text{vivo}) are also listed in this table. In control rats, the mean values of C_{isf} and C_{p,f} for probenecid from 60 to 120 min after an i.v. administration (a period of inhibition) were 25.7 ± 1.9 μM (17.4–58.7 μM, n = 23) and 199 ± 7 μM (137–254 μM, n = 24), respectively. The C_{isf/C_{p,f}} value for probenecid during a period of inhibition was significantly increased, to approximately 2- to 3-fold, by salicylate (3.67 mM) and benzoxae (3.58 mM), substrates of the MCT system, compared with that of the control. On the other hand, PAH (a substrate of the organic anion transport system) had no effect on C_{isf/C_{p,f}}. NMN (a substrate of the organic cation transport system) increased slightly, but significantly, the C_{isf/C_{p,f}} value for probenecid at the latter half of the inhibition period.

Table 4 lists the effects of probenecid, benzoxae and NMN on the C_{isf/C_{p,f}} value for salicylate. The estimated ISF concentrations of these compounds are also listed in this table. Statistically significant increases in C_{isf/C_{p,f}} were observed (approximately 1.5- to 2-fold compared with the control value) when the ISF concentrations of probenecid and benzoxae were 1.44 mM and 3.58 mM, respectively. On the other hand, NMN had little increasing effect on the C_{isf/C_{p,f}} value for salicylate.

As shown in table 5, the initial brain uptake of probenecid (or salicylate) was not increased but decreased by the intra-ISF administration of 3.58 mM salicylate (or 1.44 mM probenecid), to approximately 50% compared with the control. Furthermore, the intravascular space, measured by [14C]sucrose, did not change with the intra-ISF administration of probenecid and salicylate. These results suggest that the inhibitors neither enhance nonspecific BBB permeability nor damage the BBB.
erable damage of the BBB had occurred at 48 hr after implantation of the same type of probe used in the present study. Furthermore, at delay times beyond 3 days after probe implantation, invasion of the hypertropic astrocyte process and collagen deposition to the probe membrane have been reported (Benveniste and Diemer, 1987). These may cause unfavorable changes in the probe recovery. Therefore, the present microdialysis study was carried out at 48 hr after the surgical implantation of the probe.

The ISF-, CSF-, and brain tissue-to-plasma unbound concentration ratios of probenecid at steady state were less than unity (fig. 2A), whereas the $V_d$ value, expressing partition between the brain parenchymal tissue and the ISF, was greater than unity. These results suggest that the restricted distribution of probenecid in the brain after systemic administration may be based on mechanisms such as 1) slow penetration from blood circulation into the brain across the BBB and/or BCSFB, 2) efficient efflux transport from the brain to plasma, 3) diffusion into the CSF through the brain ISF followed by efficient efflux across the BCSFB and 4) significant metabolism in brain parenchymal cells. As shown in figure 1, the plots of ISF concentration and plasma unbound concentration never crossed throughout the period of experiment. This may suggest that a very slow rate of influx transport through the BBB is not the predominant cause of the lower levels of probenecid in ISF and brain tissue concentrations after systemic administration. The third possibility can be ruled out because of the existence of a significant

### TABLE 3

**Effect of various substrates for the specific endogenous transporters on the C_{str}/C_{p,f} ratio of probenecid**

<table>
<thead>
<tr>
<th>Time (min)$^a$</th>
<th>Control</th>
<th>Salicylate</th>
<th>Benzoate</th>
<th>PAH</th>
<th>NMM</th>
<th>Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>60–70</td>
<td>0.161 ± 0.025</td>
<td>0.328 ± 0.046**</td>
<td>0.175 ± 0.011</td>
<td>0.200 ± 0.039</td>
<td>0.141 ± 0.008</td>
<td>0.174 ± 0.012</td>
</tr>
<tr>
<td>70–80</td>
<td>0.122 ± 0.012</td>
<td>0.318 ± 0.025**</td>
<td>0.201 ± 0.007**</td>
<td>0.146 ± 0.015</td>
<td>0.160 ± 0.011</td>
<td>0.154 ± 0.008</td>
</tr>
<tr>
<td>80–90</td>
<td>0.128 ± 0.018</td>
<td>0.330 ± 0.035**</td>
<td>0.213 ± 0.028*</td>
<td>0.111 ± 0.015</td>
<td>0.160 ± 0.005</td>
<td>0.162 ± 0.018</td>
</tr>
<tr>
<td>90–100</td>
<td>0.127 ± 0.007</td>
<td>0.322 ± 0.019**</td>
<td>0.209 ± 0.003**</td>
<td>0.136 ± 0.010</td>
<td>0.196 ± 0.006**</td>
<td>0.141 ± 0.009</td>
</tr>
<tr>
<td>100–110</td>
<td>0.112 ± 0.008</td>
<td>0.323 ± 0.022**</td>
<td>0.225 ± 0.024**</td>
<td>0.134 ± 0.022</td>
<td>0.178 ± 0.008*</td>
<td>0.142 ± 0.014</td>
</tr>
<tr>
<td>110–120</td>
<td>0.119 ± 0.012</td>
<td>0.310 ± 0.020**</td>
<td>0.182 ± 0.009*</td>
<td>0.172 ± 0.020*</td>
<td>0.171 ± 0.012*</td>
<td>0.122 ± 0.008</td>
</tr>
</tbody>
</table>

$C_{str}$ denotes the inhibitor concentration in the brain ISF, estimated from the PA_{vivo} value.

*Significantly different from control ($P < .05$). **Significantly different from control ($P < .01$).

### TABLE 4

**Effect of various substrates for specific endogenous transporters on the C_{str}/C_{p,f} ratio of salicylate**

<table>
<thead>
<tr>
<th>Time (min)$^a$</th>
<th>Control</th>
<th>Salicylate</th>
<th>Benzoate</th>
<th>NMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>180–190</td>
<td>0.158 ± 0.024</td>
<td>0.227 ± 0.012</td>
<td>0.259 ± 0.007*</td>
<td>0.181 ± 0.032</td>
</tr>
<tr>
<td>190–200</td>
<td>0.166 ± 0.031</td>
<td>0.240 ± 0.015</td>
<td>0.249 ± 0.007*</td>
<td>0.175 ± 0.017</td>
</tr>
<tr>
<td>200–210</td>
<td>0.150 ± 0.022</td>
<td>0.238 ± 0.016**</td>
<td>0.273 ± 0.012**</td>
<td>0.186 ± 0.010</td>
</tr>
<tr>
<td>210–220</td>
<td>0.159 ± 0.019</td>
<td>0.238 ± 0.003**</td>
<td>0.286 ± 0.008**</td>
<td>0.189 ± 0.019</td>
</tr>
<tr>
<td>220–230</td>
<td>0.166 ± 0.026</td>
<td>0.253 ± 0.014**</td>
<td>0.280 ± 0.008**</td>
<td>0.181 ± 0.008</td>
</tr>
<tr>
<td>230–240</td>
<td>0.159 ± 0.033</td>
<td>0.267 ± 0.007**</td>
<td>0.269 ± 0.008**</td>
<td>0.170 ± 0.004</td>
</tr>
<tr>
<td>240–250</td>
<td>0.153 ± 0.034</td>
<td>0.306 ± 0.033**</td>
<td>0.304 ± 0.012**</td>
<td>0.183 ± 0.009</td>
</tr>
<tr>
<td>250–260</td>
<td>0.147 ± 0.033</td>
<td>0.298 ± 0.028**</td>
<td>0.278 ± 0.010**</td>
<td>0.175 ± 0.007</td>
</tr>
<tr>
<td>260–270</td>
<td>0.155 ± 0.026</td>
<td>0.323 ± 0.025**</td>
<td>0.270 ± 0.009*</td>
<td>0.189 ± 0.017</td>
</tr>
</tbody>
</table>

$C_{str}$ denotes the inhibitor concentration in the brain ISF, estimated from the PA_{vivo} value.

*Significantly different from control ($P < .05$). **Significantly different from control ($P < .01$).

### TABLE 5

**Effect of inhibitors on the initial uptake of probenecid and salicylate and on sucrose space in the brain**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Probenecid</th>
<th>Salicylate</th>
<th>[^1C]Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0898 ± 0.0231</td>
<td>0.0756 ± 0.0042</td>
<td>0.0286 ± 0.0027 (HP)</td>
</tr>
<tr>
<td>Probenecid</td>
<td>—</td>
<td>0.0532 ± 0.0013**</td>
<td>0.0310 ± 0.0042 (RH)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>0.0481 ± 0.0052</td>
<td>—</td>
<td>0.0209 ± 0.0071 (HP)</td>
</tr>
</tbody>
</table>

[^1C]Sucrose denotes the inhibitor concentration in the brain ISF, estimated from the PA_{vivo} value.

*Significantly different from control ($P < .05$). **Significantly different from control ($P < .01$).
downhill concentration gradient from the CSF pool toward the ISF space at the steady state. In addition, the major elimination pathway of probenecid in rats has been reported to be hepatic metabolism, by glucuronidation of probenecid and its hydroxypropyl derivatives (Conway and Melethil, 1974); this makes the fourth explanation improbable. Accordingly, it is likely that efficient efflux from the brain ISF across the BBB is responsible for the restricted distribution of probenecid in the brain.

To support the above hypothesis, we first tried to determine, by the intra-ISF administration method, whether probenecid is actually transported from brain ISF to plasma across the BBB. The concentrations of probenecid in the brain ISF around the microdialysis probe declined more rapidly than those of [14C]MeAIB, which was used as a diffusion marker in the brain parenchymal tissue (fig. 4A and B). These results clearly suggest the efflux of probenecid via the BBB. We used [14C]MeAIB as a diffusion marker because 1) the blood-to-brain transport clearance of this compound has been reported to be very small (0.0057 ml/min/g brain) (Blasberg et al., 1983), and 2) rapid efflux of [14C]MeAIB across the BBB has not been proved, although this compound can be actively taken up by isolated cerebral capillaries (Betz and Goldstein, 1978). The fairly good coincidence between the apparent disappearance rate of [14C]MeAIB in the brain ISF and that in agar gel (fig. 4B), suggests that the brain-to-plasma efflux of [14C]MeAIB is practically negligible in the present experimental condition. In addition, the apparent disappearance rate of probenecid in agar gel was comparable with that of [14C]MeAIB, which suggests that the diffusion rate of probenecid through the brain ISF approximates that of [14C]MeAIB.

Second, we estimated CL_{in} and CL_{out} according to equations (2) and (7) to evaluate quantitatively the BBB transport of probenecid. The CL_{in} value, estimated by the in situ brain perfusion technique (table 2), was remarkably smaller than the cerebral plasma flow (0.515 ml/min/g brain; Sakurada et al., 1978). The validity of this value was checked by an integration plot analysis of the initial uptake by the brain after an i.v. administration of probenecid (equation 4). The value estimated was in good agreement with that obtained by the in situ brain perfusion. Additionally, the V_i value approximated the intravascular space in the brain microvasculature, which suggests that the penetration from plasma across the BBB is a rate-limiting step for distribution into the brain. On the other hand, the CL_{out} value for probenecid, estimated by equation (7), was significantly greater than the CL_{in} value and the convective flow in ISF (Ohno et al., 1978). If the influx clearance of probenecid is not saturated with the high concentration in the intravascular space, then the kinetic finding (CL_{out} > CL_{in}) would suggest that the BBB may have an asymmetric and active nature in the transport of probenecid.

Recent findings using cultured BCECs have demonstrated that acidic drugs, like salicylate, are transported from the luminal side into brain by the MCT system (Terasaki et al., 1991). Further, the gene expression of a monocarboxylate transporter, MCT1, at the BBB of rats has been confirmed by reverse transcriptase-polymerase chain reaction of poly(A) RNA derived from rat brain capillaries (Takanaga et al., 1995). This transporter is homologous with MCT1 isolated in Chinese hamster ovary cells (Garcia et al., 1994). In contrast to these findings, it remains unknown whether the efflux of salicylate from the brain to plasma is mediated by the MCT system. However, the present findings shown in figure 2 and table 2 suggest that salicylate is efficiently transported across the BBB from the brain to plasma. To obtain further in vivo evidence bearing on this issue, we attempted inhibition studies by benzoate, an alternative compound that is recognized by the MCT system (Tsuji et al., 1994). As shown in table 4, C_{isf/Cp,f} was significantly increased by benzoate (3.58 mM). Taking into account the K_i value (4.50 mM) of benzoate estimated from the report on the MCT system in cultured BCECs (Terasaki et al., 1991), the brain-to-plasma efflux of salicylate should be inhibited by benzoate, thus resulting in the increased C_{isf/Cp,f}. On the other hand, an organic cation, NMN, did not produce increases in the C_{isf/Cp,f} value for salicylate. These results suggest that salicylate and benzoate may be transported from the brain ISF to plasma across the BBB, presumably by the MCT system.

Interestingly, the C_{isf/Cp,f} value for probenecid was significantly increased by salicylate (3.67 mM; table 3), which suggests that the efflux transport of probenecid at the BBB can mutually interact with that of salicylate. Recently, Loike et al. (1993) demonstrated that L-lactate uptake into mouse peritoneal macrophages occurs via a probenecid-inhibitable MCT system that is distinct from the anion exchange system. In addition, it has been reported that probenecid can block competitively both the efflux of lactate from parenchymal cells to the ISF and the consequent reuptake by cells in the striatum of rats (Kuhr et al., 1988). These findings suggest the possibility that probenecid itself is transported by the MCT system.

The inhibition studies on the efflux transport of probenecid at the BBB were undertaken to investigate the above hypothesis. As shown in table 4, the C_{isf/Cp,f} value for probenecid was increased by not only salicylate (3.67 mM) but also benzoate (3.58 mM), and their increased effects were sustained over a period of inhibition. Moreover, given the lack of an enhancing effect on the initial brain uptake of probenecid, and the absence of damage of the BBB, by salicylate (table 5), the decrease in CL_{out} by their inhibitors may be responsible for the increases in the C_{isf/Cp,f} ratio for probenecid. On the basis of the K_i values of salicylate (3.60 mM) and benzoate (4.50 mM) for the MCT system in cultured BCECs (Terasaki et al., 1991), the increase in the C_{isf/Cp,f} ratio for probenecid brought about by these inhibitors can be calculated according to a competitive inhibition model based on the simple Michaelis-Menten equation. Approximately a 2-fold increase in the C_{isf/Cp,f} ratio for probenecid was predicted from the inhibition by salicylate and benzoate. This estimate corresponded well with the experimental results. In addition, we found that NEM treatment increased the C_{isf/Cp,f} value for probenecid, whereas CL_{in} remained unchanged. A recent report (Loike et al., 1993) that L-[14C]lactate uptake via a probenecid-inhibitable MCT-1 system in macrophages is inhibited by NEM may support the interpretation that probenecid is transported out of the brain via the MCT system at the BBB.

In contrast to the above results, PAH and choline showed little increasing effect on the C_{isf/Cp,f} ratio for probenecid (table 3). Probenecid is known to be a substrate of the organic anion transport system, just as PAH is, with a K_m value of 40 μM and a K_v value of 500 μM (Sheikh and Maxild, 1978) for
PAH. Recently, sophisticated work by Kakee et al. (1995) has demonstrated that a tracer level of PAH is pumped from the cerebrum via the organic anion transport system and that its transport is completely inhibited by probenecid. However, the present study failed to find inhibitory effects by PAH. One possible explanation for this discrepancy is that saturation of the organic anion transport system at the higher ISF concentration of probenecid and insufficent concentration of PAH as an inhibitor may result in the lack of inhibitory effect by PAH.

It is of interest that the $C_{in}/C_{pl}$ value for probenecid was significantly increased, to approximately 1.5-fold, by NMN at the latter phase of the inhibition period. Because probenecid competitively inhibits NMN transport with a $K_m$ value of 2.01 $\mu M$ in the renal proximal tubule (Hayu et al., 1988), the organic cation transport system may contribute to the efflux of probenecid at the BBB. Further detailed study will be necessary to investigate this possibility.

In conclusion, the restricted distribution of probenecid in the brain may be ascribed to efficient efflux from the brain ISF across the BBB, which may be mediated by the MCT system at a relatively high ISF concentration of probenecid. This study also indicates that the previously suggested probenecid-sensitive efflux system may include several transport systems, such as MCT and NMN-sensitive organic cation transport systems, as well as an organic anion transport system in common with PAH, which has been previously defined by other investigators. Therefore, probenecid would increase the brain concentration by inhibiting the BBB efflux of drugs that are at least recognized by these efflux systems, which would enhance the CNS effect and minimize the therapeutic dose and the systemic toxicity. For example, it has been reported that baclofen inhibits the presynaptic release of amino acids via GABA$_A$ receptor (Losada and Acosta, 1992). However, this effect requires a relatively high concentration in ISF (> 10 $\mu M$). Our recent finding (Deguchi et al., 1995) in rats has shown that the coadministration of baclofen with probenecid reduces the efflux clearance of baclofen at the BBB, resulting in a significant increase in ISF/plasma ratios without a substantial reduction of the renal clearance. Therefore, the use of probenecid as an adjunct to baclofen might develop a potential CNS effect to improve brain function. As this example illustrates, the present study would provide significant information for drug therapy and development of an efficient delivery system for administering several centrally acting pharmaceuticals to the brain.

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References


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