Hydrogen Bonding Potential as a Determinant of the *in Vitro* and *in Situ* Blood–Brain Barrier Permeability of Peptides

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With the exception of various central nervous system (CNS)-required nutrients for which specific, saturable transport systems exist, the passage of most water-soluble solutes through the blood–brain barrier (BBB) is believed to depend largely on the lipid solubility of the solutes. Most peptides, therefore, do not enter the CNS because of their hydrophilic character. Recently, utilizing homologous series of model peptides and Caco-2 cell monolayers as a model of the intestinal mucosa, it was concluded that the principal determinant of peptide transport across the intestinal cellular membrane is the energy required to desolvate the polar amide bonds in the peptide (P. S. Burton *et al., Adv. Drug Deliv. Rev.* 7:365–386, 1991). To determine whether this correlation can be extended to the BBB, the permeabilities of the same peptides were determined using an *in vitro* as well as an *in situ* BBB model. The peptides, blocked on the N- and C-terminal ends, consisted of D-phenylalanine (F) residues: $AcFNH_2$, AcF_2NH_2, AcF_3NH_2, $AcF_2(NMeF)NH_2$, $AcF(NMeF)_2NH_2$, $Ac(NMeF)_3NH_2$, and $Ac(NMeF)_3NHMe$. A good correlation among the permeabilities of these model peptides across the bovine brain microvessel endothelial cell (BBMEC) monolayers, an *in vitro* model of the BBB, and their permeabilities across the BBB *in situ* was observed ($r = 0.928$, $P < 0.05$). The permeabilities of these peptides did not correlate with the octanol–buffer partition coefficients of the peptides ($r = 0.389$ *in vitro* and $r = 0.155$ *in situ*; $P < 0.05$). However, correlations were observed between the permeabilities of these peptides and the number of potential hydrogen bonds the peptides can make with water ($r = 0.837$ *in vitro* and $r = 0.906$ *in situ*; $P < 0.05$), suggesting that desolvation of the polar bonds in the molecule is a determinant of permeability. Consistent with this, good correlations were found between the permeabilities of these peptides and their partition coefficients between heptane–ethylene glycol ($r = 0.981$ *in vitro* and $r = 0.940$ *in situ*; $P < 0.05$) or the differences in partition coefficients between octanol–buffer and isooctane–buffer (ΔlogPC) ($r = 0.961$ *in vitro* and $r = 0.962$ *in situ*; $P < 0.05$), both of which are experimental estimates of hydrogen bond or desolvation potential. These results suggest that the permeability of peptides through the BBB is governed by the same physicochemical parameter (hydrogen bonding potential) as their permeability through the intestinal mucosa.

KEY WORDS: hydrogen bonding potential; desolvation energy; blood–brain barrier; *in situ* rat brain perfusion; bovine brain microvessel endothelial cells; peptide transport; *in vitro* and *in situ* correlation.

INTRODUCTION

The blood–brain barrier (BBB), which functions to ensure that the homeostasis of the brain is maintained, is the major obstacle for the development of centrally active peptides as neuropharmaceuticals. Specialized characteristics of the endothelial cells that form the brain capillaries are responsible for this barrier (1,2). Brain capillary endothelial cells are joined together by tight intercellular junctions that, combined with their minimal endocytotic activity, form a continuous barrier against the movement of bloodborne solutes from blood to brain (1–5). With the exception of various water-soluble nutrients, which are transported across the BBB by specific carrier systems, passage of most bloodborne solutes through the BBB is dependent largely on their lipid solubility (6,7). Most peptides are very hydrophilic and are, therefore, believed not to enter the central nervous system (CNS) (6,7).

Recently, utilizing Caco-2 cell monolayers as a model of the intestinal mucosa, systematic studies of the influence of peptide structure on membrane permeability have been reported (8–10). Employing model peptides, which had systematic modifications in their structures, the contributions of various physicochemical parameters (molecular weight, lipophilicity, and hydrogen bonding potential) of these peptides on their permeability were explored. The major observation from these studies was that the permeabilities of these peptides across Caco-2 cells was found to correlate better with the number of potential hydrogen bonds the peptide could form with water. This correlation was rationalized in the context of a model in which the energy required to desolvate the peptide polar functional groups for it to enter and diffuse across the cell membrane was a principal determinant of permeability (8–10). Consistent with these ideas, good correlations of Caco-2 cell permeability were reported with experimental determined estimates of hydrogen bond potential. Specifically, desolvation energy for the peptides was approximated either by calculating the difference between the partition coefficients of the peptides obtained in the octanol–buffer partitioning system and isooctane–buffer partitioning system (ΔlogPC) modeled after earlier work by Seiler (11) or by the partition coefficients of the peptides obtained in the heptane–ethylene glycol partitioning system (12).

In an attempt to determine whether the desolvation energy of a peptide can be used to predict its BBB transport, the permeabilities of these same model peptides (8–10) across an *in vitro* and an *in situ* model of the BBB were determined. The *in vitro* model of the BBB consists of primary culture of bovine brain microvessel endothelial cell (BBMEC) monolayers, which have been shown to retain many of the *in vivo* characteristics of the BBB (13). For the *in situ* BBB model, an *in situ* rat brain perfusion technique originally developed by Takasato *et al.* (14) was utilized. This technique has been shown to allow accurate quantitation of solute uptake into the brain while maintaining the BBB integrity during the perfusion. Our results indicated that the BBB permeabilities of the model peptides were inversely correlated with their respective estimates of desolvation energy, suggesting that increased transport of peptides across

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the BBB can be achieved by reduction of the desolvation energies of the peptides. In addition, the good correlation between the *in vitro* and the *in situ* permeabilities of the model peptides obtained in our studies suggests the feasibility of using the *in vitro* model of the BBB to predict the *in vivo* BBB permeability of peptides.

MATERIALS AND METHODS

Animals

Ten- to twelve-week-old Sprague–Dawley male rats (Sasco, Omaha, NE), weighing between 300 and 400 g, were used in the *in situ* rat brain perfusion experiments.

Peptides

The model peptides [$AcFNH_2$, **1**; AcF_2NH_2, **2**; AcF_3NH_2, **3**; $AcF_2(NMeF)NH_2$, **4**; $AcF(NMeF)_2NH_2$, **5**; $Ac(NMeF)_3NH_2$, **6**; $Ac(NMeF)_3NHMe$, **7**] were synthesized and radiolabeled with ^{14}C in the acetamide [*C1*] carbon position, having specific activities of approximately 110 μCi/μmol (8,9). The peptides were stored in a CH_3OH:$CHCl_3$ (50:50) solution at −70°C before use. All other reagents were analytical grade.

In Vitro Model of the BBB

Cell Culture Studies

The BBMECs were isolated from cerebral gray matter of fresh bovine brains following the procedure originally described by Audus and Borchardt (13) with a slight modification. This modification involves filtering the BBMEC obtained from the Percoll centrifugation step of the original procedure through a sequence of polypropylene meshes (mesh size, 297 and 105 μm). This modification resulted in an increase in the tightness of the cell monolayer, thus further improving the *in vitro* system to mimic the *in vivo* BBB (15). Approximately 235,000 BBMECs were seeded on a type I collagen-coated (Collaborative Research, Lexington, MA) and bovine fibronectin-treated (Sigma Chemical Co., St Louis, MO) microporous membrane in the Costar Transwell (24 mm in diameter, 3.0-μm pore size). The cells were cultured using 2.6 mL of culture medium in the basolateral (BL) side and 1.5 mL in the apical (AP) side. The culture medium, which consisted of modified Eagle medium (MEM):F12 (1:1) supplemented with 10% heat-inactivated horse serum (Intergen, Purchase, NY) and 125 μg/mL heparin (Sigma Chemical Co., Cat. No. H-3125), was changed every other day. The cells were cultured at 37°C with 95% humidity and 5% CO_2 and reached confluency in about 7 to 8 days. After 10 days of culturing, to increase further the tightness of the cell monolayer integrity (16), the BBMECs were treated with culture medium containing 250 μ*M* 8-(−4-chlorophenylthio) cAMP (Boehringer Mannheim, Indianapolis, IN) plus a 17.5 μ*M* concentration of the phosphodiesterase inhibitor RO20-1724 (BioMol, Plymouth Meeting, PA) for 1 to 2 days and then used for transendothelial transport experiments.

Transport Studies

BBMECs were preincubated at 37°C in pH 7.4 buffer containing 122 m*M* NaCl, 25 m*M* $NaHCO_3$, 10 m*M* D-glucose, 3 m*M* KCl, 2.5 m*M* $MgSO_4$, 0.4 m*M* K_2HPO_4, 1.4 m*M* $CaCl_2$, 10 m*M* HEPES, 250 μ*M* 8-(−4-chlorophenylthio) cAMP, and a 17.5 μ*M* concentration of the phosphodiesterase inhibitor RO20-1724 (transport buffer) for 15 to 30 min. Prior to the transendothelial transport studies, the transport buffer in both the AP and the BL sides was aspirated. Fresh transport buffer (2.6 mL) was then added to the BL side. To start the transport experiment, 1.5 mL of transport buffer containing either 0.1 μCi/mL of [^{14}C]sucrose (sp act, 63.2 μCi/μmol; NEN Research Products, DuPont Company, Wilmington, DE), an impermeant marker, or 0.1 μCi/mL of the model peptides was added to the AP side. Samples (50 μL) were then taken from the receiver side (BL) at 15, 30, 45, 60, and 90 min and replaced with an equal volume of transport buffer. The amount of radiolabeled compounds on the receiver side was then determined in a Beckman LS 6000 IC liquid scintillation counter. The sucrose leakage across the BBMEC monolayers was ~5% per hour.

Determination of Permeability Coefficients ($P_{monolayer}$) of Model Peptides Across BBMEC Monolayers

The apparent permeability coefficients ($P_{app:cell}$) for model peptides **1** to **7** across BBMEC monolayers grown on Transwell collagen-coated and fibronectin-treated polycarbonate membranes were calculated according to the following equation:

$$P_{app:cell} = flux_{monolayer} \times (1/A) \times (1/C) \quad (1)$$

where C is the initial peptide concentration in the AP side (pmol/mL), A is the surface area of the Transwell (cm^2), and $flux_{monolayer}$ is the steady-state rate of appearance of apically applied radiolabeled peptides on the BL side of the monolayers (pmol/sec) after the initial lag time for the peptides to appear on the BL side. $P_{app:cell}$ reflects the permeability of the peptides through the aqueous boundary layer adjacent to the surface of the cell monolayer ($P_{ABL:cell}$), across the cell monolayer ($p_{monolayer}$), through the collagen-coated and fibronectin-treated polycarbonate membrane (P_{filter}), and through the aqueous boundary layer adjacent to the bottom surface of the polycarbonate membrane ($P_{ABL:membrane}$). Since these parameters are resistant to diffusion in series, they are related by the following expression (17):

$$1/P_{app:cell} = (1/P_{ABL:cell}) + (1/P_{monolayer}) + (1/P_{filter}) + (1/P_{ABL:membrane}) \quad (2)$$

Upon rearrangement, this yields Eq. (3), which should allow $P_{monolayer}$ to be calculated if the values of the other three parameters ($P_{ABL:cell}$, P_{filter}, and $P_{ABL:membrane}$) are known.

$$P_{monolayer} = 1/[(1/P_{app:cell}) - (1/P_{ABL:cell}) - (1/P_{filter}) - (1/P_{ABL:membrane})] \quad (3)$$

Like $1/P_{app:cell}$, the reciprocal of the apparent permeability coefficients for model peptides **1** to **7** across the collagen-coated and fibronectin-treated polycarbonate membrane Transwell ($1/P_{app:filter}$) can be determined and written as the sum of the reciprocals of three permeability parameters:

$$1/P_{app:filter} = (1/P_{ABL:filter}) + (1/P_{filter}) + (1/P_{ABL:membrane}) \quad (4)$$

where $P_{ABL:filter}$ and $P_{ABL:membrane}$ reflect the permeability of the peptide through the aqueous boundary layers adjacent to the surface of the collagen-coated and fibronectin-treated polycarbonate membrane and the other surface of the polycarbonate membrane, respectively. Upon rearrangements, Eq. (4) yields Eq. (5):

$$1/P_{filter} = (1/P_{app:filter}) - (1/P_{ABL:filter}) - (1/P_{ABL:membrane}) \quad (5)$$

which, when combines with Eq. (3), yields

$$P_{monolayer} = 1/[(1/P_{app:cell}) - (1/P_{ABL:cell}) - (1/P_{app:filter}) + (1/P_{ABL:filter})] \quad (6)$$

The effects of the aqueous boundary layers adjacent to the cell monolayer ($P_{ABL:cell}$) and the collagen-coated and fibronectin-treated polycarbonate membrane ($P_{ABL:filter}$) on the permeability of model peptides can be approximated by the $P_{ABL:cell}$ and $P_{ABL:filter}$ values of [^{14}C]testosterone (sp act, 55.4 μCi/mmol; NEN Research Products, DuPont Company, Wilmington, DE) which were determined in previous experiments (18). Our data indicated that there is essentially no difference between the $P_{ABL:cell}$ and $P_{ABL:filter}$ values. Thus, based on this approximation, Eq. (6) was further simplified to become

$$P_{monolayer} = 1/[(1/P_{app:cell}) - (1/P_{app:filter})] \quad (7)$$

which gives the permeability coefficient of model peptides across BBMEC monolayers as the reciprocal of the difference between $1/P_{app:cell}$ and $1/P_{app:filter}$, the two parameters which were both determined experimentally.

In Situ Model of the BBB: Rat Brain Perfusion Technique

The transport of model peptides across the BBB was quantified in pentobarbital-anesthetized rats (50 mg/kg, i.p.) using the *in situ* rat brain perfusion technique developed by Takasato *et al.* (14). The right pterygopalatine, occipital, and superior thyroid arteries were coagulated and cut, and the right common carotid artery was prepared for ligation by loosely encircling the artery with surgical silk just proximal to the bifurcation of the external carotid artery. A catheter (PE-50) filled with heparinized saline (100 IU/mL) was then placed in the right external carotid artery for retrograde perfusion. The rectal temperature was maintained at 36.5 ± 0.5°C throughout the experiment by a heat lamp connected to a feedback device (YSI Model 73 ATD indicating controller). Prior to perfusion, the perfusate was filtered, oxygenated with 95% air/5% CO_2, and heated to 37°C. The perfusate consisted of pH 7.4 bicarbonate-buffered physiologic saline (128 m*M* NaCl, 24 m*M* $NaHCO_3$, 4.2 m*M* KCl, 2.4 m*M* NaH_2PO_4, 1.5 m*M* $CaCl_2$, 0.9 m*M* $MgCl_2$, and 9 m*M* D-glucose) containing ^{3}H-labeled methoxy inulin (187 mCi/g; NEN), a vascular marker, and the ^{14}C-labeled model peptide at a ratio of between 3:1 and 10:1.

Immediately after performing a heart-cut on the anesthetized rat and ligating the right common carotid artery, perfusate was perfused by an infusion pump (Model 355 syringe pump, Saga Instruments, Cambridge, MA) at a flow rate of 4.5 mL/min into the right hemisphere of the rat brain via a syringe connected to the catheter in the right external carotid artery. After a period of 60, 120, 180, or 240 sec, the perfusion was terminated by decapitation of the animal.

The rat brain was removed from the skull and dissected on ice following the procedure of Ohne *et al.* (19). A brain tissue sample (20 to 30 mg) from the parietal cortex of the right cerebral hemisphere was weighed and digested overnight at 50°C in 1 mL water containing 10% piperidine. After overnight digestion, the sample was counted for radioactivity using a dual-label scintillation spectrometer (Beckman LS 6000 IC). To determine the original radioactivity level of the radiolabeled tracers in the perfusate, 30-μL samples of the perfusion fluid were also collected, digested, and counted.

Determination of Parietal Cerebrovascular Permeability Coefficients ($P_{in\ situ}$) of the Model Peptides

The transport of the model peptides across the BBB was expressed in terms of three parameters: the parietal unidirectional blood-to-brain transfer constants (K_{in}), the parietal cerebrovascular permeability–surface area products (*PA*), and the parietal cerebrovascular permeability coefficients ($P_{in\ situ}$) of the model peptides (20). The K_{in} for the model peptides was calculated from the quantity of radiolabeled model peptide taken up into the parietal region of the cerebral cortex using the following equation:

$$K_{in} = (q^*_{tot} - V_v C^*_{pf})/TC^*_{pf} \quad (8)$$

where q^*_{tot} is the total measured ^{14}C-radioactivity in the parietal region of the cerebral cortex (dpm/g), V_v is the intravascular volume (mL/g) in this same region, C^*_{pf} is the model peptide concentration in the perfusion fluid (dpm/mL), and T is the net perfusion time (sec). V_v was determined in each animal from the brain-to-perfusion fluid ratio of [^{3}H]methoxy inulin, a vascular marker (14), using the following equation:

$$V_v(\%) = 100\ ([^3H]\ dpm \cdot g^{-1})/([^3H]\ dpm \cdot mL\ HCO_3\ saline^{-1}) \quad (9)$$

Brain perfusion times were chosen so that two important criteria for generation of reliable permeability coefficients would be met (14,21). First, the perfusion time should be long enough to ensure that at least 50% of the peptide in the total brain sample (brain tissue and vascular volume) is present in the brain tissue ($q^*_{tot} - V_v C^*_{pf} > 0.5\ q^*_{tot}$). Second, the perfusion time should be limited to ensure that the peptide concentration in the brain tissue is less than 20% of the peptide concentration in the perfusate ($q^*_{tot} - V_v C^*_{pf} < 0.2\ C^*_{pf}$) to prevent any significant backflux in the brain-to-blood direction. The integrity of the BBB as a function of perfusion time was determined by measuring the extent of the brain accumulation of [^{14}C]urea (56 μCi/μmol; NEN), a marker for BBB integrity.

K_{in} values were converted to *PA* products using the Crone–Renkin model of capillary transfer (22).

$$PA = -F \ln[(1 - K_{in})/F] \quad (10)$$

where F is the regional perfusion fluid flow (mL/sec/g). F was measured using ^{3}H-labeled diazepam (14) and was determined in a separate set of experiments to be 7.28 ± 0.45

$\times 10^{-2}$ mL/sec/g in the parietal cortex ($n = 4$). P (cm/sec) was calculated by dividing PA by $A = 130$ cm^2/g (23).

HPLC Analysis of Radiolabeled Model Peptides

To assure that the ^{14}C-radioactivity at the BL side of the BBMEC monolayers and in the rat brain samples corresponded to that of intact parent peptides, it was necessary to show that no metabolism had taken place during BBB transport. At the completion of the *in vitro* transport experiments, samples (50 μL) were taken basolaterally and analyzed by HPLC as described previously (8,9). To demonstrate the metabolic stability during the *in situ* rat brain perfusion, model peptides at concentrations between 2 and 9 μmol/L were incubated with rat brain homogenate (20%, w/v, brain in bicarbonate-buffered physiologic saline) at 37°C for 30 min. Samples of 0.5-mL brain homogenate were withdrawn at 10-min time intervals and mixed with 1.0 mL cold acetonitrile to precipitate the proteins. Each sample was then centrifuged (Fisher Scientific Marathon 21 K/BR) at 13,000 rpm for 30 min at 4°C and the resulting supernatant was filtered (Millipore FH 0.5 μm) and analyzed by HPLC.

The HPLC system consisted of a Beckman pump (112 Solvent Delivery Module) and a Perkin-Elmer LCI-100 integrator. The column was a Brownlee Sphere-5 RP-18 (5 μm, 4.6 × 100 mm) with a Brownlee Sphere-5 RP-18 guard column (5 μm, 4.6 × 100 mm). The mobile phase consisted of 40% acetonitrile, 60% distilled water, 0.02% trifluoroacetic acid (TFA), and 0.02% dimethyloctylamine (DMOA). The flow rate was 1.0 mL/min at room temperature. Samples (50 μL) were injected into the HPLC system and the effluent fractions were collected at 0.2-min time intervals using a Bio-Rad (Model 2110) fraction collector. Three milliliters of scintillation cocktail (3a70BTM from RPI Corp., Mount Prospect, IL) was then added to 150 μL of the collected fractions and the ^{14}C-radioactivity was determined by liquid scintillation counting (Beckman LS 6000 IC).

Statistical Analysis

All correlation coefficients (r) were obtained by linear regression analysis. The statistical significance of each correlation coefficient was determined using the Student t test at $P < 0.05$ (24,25). A two-way t test ($P < 0.05$) involving an r-to-z-to-z^* Hotelling's transformation (24,25) was used to analyze the statistical significance of differences between correlation coefficients.

RESULTS AND DISCUSSION

To determine the effects of hydrogen bonding potential on the BBB permeability of peptides, the permeability of a series of model peptides consisting of D-phenylalanine (F) residues across an *in vitro* and *in situ* model of the BBB was studied. These peptides, varying in chain length, lipophilicity, and hydrogen bond number, are also expected to be metabolically inert due to the use of the unnatural D-amino acids as has been described previously (8,9).

Figure 1 shows the time course of the cumulative appearance of the model peptides in the BL side of the BBMEC monolayers after their AP applications. The flux of the peptides appears to be linear during the time course of the study. To confirm that the radioactivity measured was associated with the parent model peptides and not metabolized fragments of the parent peptides, HPLC analysis was performed. At the beginning and completion of the *in vitro* transport experiments, aliquots of the donor (AP) and receiver (BL) compartments for two representative peptides (3 and 5) were taken and analyzed by HPLC. Our results indicated that no apparent metabolism of peptide took place during its transit from the AP to the BL side (data not shown). *In vitro* permeability coefficients ($P_{monolayer}$) for the model peptides were then calculated from the steady-state rate of appearance of the peptides as described under Materials and Methods and are summarized in Table I. It should be pointed out that, since this model shows a fairly high flux of sucrose, these permeability coefficients contain contributions from paracellular as well as transcellular flux. However, as discussed previously with respect to the Caco-2 cell studies with these peptides (8), this will be more significant for the least permeable solute (3) and less important for the more permeable peptides (6 and 7 for example).

When the *in situ* rat brain perfusion technique was used

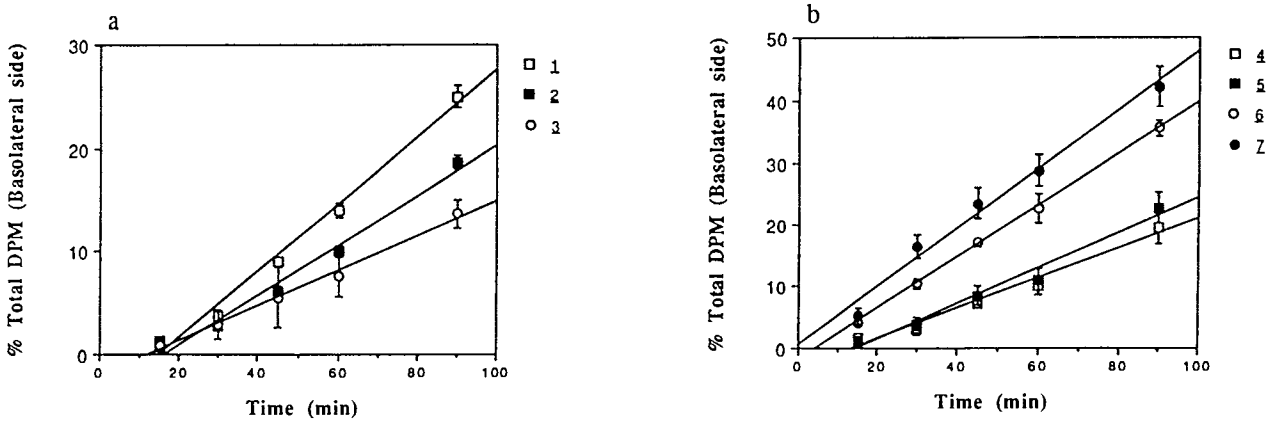

Fig. 1. Flux of model peptides 1–3 (a) and 4–7 (b) across confluent BBMEC monolayers. $AcFNH_2$ (1), AcF_2NH_2 (2), AcF_3NH_2 (3), $AcF_2(NMeF)NH_2$ (4), $AcF(NMeF)_2NH_2$ (5), $Ac(NMeF)_3NH_2$ (6), and $Ac(NMeF)_3NHMe$ (7) were added to the AP side of the BBMEC monolayers that were previously incubated in transport buffer at 37°C. Each point represents the mean and standard deviation for three determinations.

Table I. Physicochemical and Permeability Properties of the Model Peptides 1–7

Peptide	MW	No. of hydrogen bonds	log*PC*[a] octanol–buffer	Δlog*PC*[a,b]	log*PC*[a] heptane–ethylene glycol	$P_{monolayer}$ [10^5 cm sec^{-1} (SD)][c]	$P_{in\ situ}$ [10^7 cm sec^{-1} (SD)][c]
$AcFNH_2$ (1)	206	5	0.05	4.97	−5.46	3.69 (0.60)	18.29 (2.97)
$AcFFNH_2$ (2)	353	7	1.19	6.48	−6.52	2.16 (0.14)	1.69 (0.54)
$AcFFFNH_2$ (3)	500	9	2.30	7.32	−7.10	1.28 (0.39)	0.87 (0.29)
$AcFF(Me)FNH_2$ (4)	514	8	2.63	6.83	−6.28	2.20 (0.46)	2.08 (0.42)
$AcF(Me)F(Me)FNH_2$ (5)	528	7	2.53	5.63	−5.14	3.06 (0.57)	6.92 (1.75)
$Ac(Me)F(Me)F(Me)FNH_2$ (6)	542	6	2.92	4.59	−4.20	7.59 (0.83)	10.85 (1.50)
Ac(Me)F(Me)F(Me)FNH(Me) (7)	556	5	3.24	3.93	−2.86	12.95 (4.84)	113.71 (49.4)

[a] Partition coefficient values (PC) were taken from Refs. 10 and 12.
[b] $\Delta logPC$ = log*PC*(octanol–buffer) − log*PC*(isooctane–buffer).
[c] Permeability coefficient values ($P_{monolayer}$) in BBMEC monolayers and parietal permeability coefficient values ($P_{in\ situ}$) in the *in situ* rat brain perfusion technique were determined as described under Materials and Methods. Each value represents the mean ± SD of at least three determinations.

to study the BBB permeability of the model peptides into the parietal cortex using a perfusion time of 60 sec, model peptides **2**, **3**, **4**, and **5** exhibited very low BBB permeabilities. As a result, their levels in the brain tissue were below the 50% criterion described under Materials and Methods. Therefore, the permeability coefficients obtained for these model peptides were not reliable. One way to increase the levels of these peptides in the brain tissue was to increase the perfusion time. It was found in our subsequent experiments that perfusion times of up to 240 sec were required for 50% of peptides **4** and **5** to appear in the brain tissue. However, despite a perfusion time of 240 sec, only 40 and 25% of peptides **2** and **3**, respectively, were shown to be present in the brain tissue. Although the obtained permeability coefficients for peptides **2** and **3** are less reliable, perfusion times could not be extended further because perfusing the brain for longer periods of time may increase the chance of breaching the BBB integrity. The integrity of the BBB was shown to be maintained for perfusion times of up to 240 sec. This was done by determining the unidirectional blood-to-brain transfer constant (K_{in}) of [^{14}C]urea, a marker for determining BBB integrity. Using perfusion times of up to 240 sec, the K_{in} of urea was determined to be 6.95×10^{-5} mL/sec/g. This value was comparable to the previously reported K_{in} value ($7.6 \pm 0.8 \times 10^{-5}$ mL/sec/g) of urea across the intact BBB (26).

To test the metabolic stability of the peptides while in contact with enzymes in the rat brain, the peptides were incubated with 20% fresh rat brain homogenate at 37°C for 30 min and were HPLC analyzed. All peptides were shown to be stable under these conditions for at least 30 min (data not shown). Since the peptides are perfused into the rat brain only for up to 240 sec, it was concluded that no significant metabolism of the model peptides would take place during the perfusion and while the peptides cross the BBB *in situ*.

Table I lists the permeability coefficients ($P_{monolayer}$) and the parietal cerebrovascular permeability coefficients ($P_{in\ situ}$) for model peptides 1–7, along with their respective physicochemical parameters. It can be seen that as the chain length in the model peptides **1–3** increases, a decrease in the permeability coefficients ($P_{monolayer}$ and $P_{in\ situ}$) was observed despite the corresponding increases in their lipophilicities as measured by the log partition coefficient of octanol–buffer. However, a significant increase in the permeability coefficients, from 1.28×10^{-5} to 12.95×10^{-5} cm/sec for $P_{monolayer}$ and from 0.87×10^{-7} to 113.71×10^{-7}/sec for $P_{in\ situ}$, was observed as model peptide **3** was sequentially methylated (analogue **4–7**). In this series, the chain length is constant and the octanol/buffer partition coefficient changes only slightly and in an irregular fashion, while the hydrogen bond number decreases regularly with each methyl group introduced.

These results are similar to those obtained earlier in the Caco-2 cell model (8–10) and support the idea that hydrogen bond number is important in determining peptide permeability across the BBB as well. This conclusion is further supported by the relatively good correlation coefficients (r = 0.837 *in vitro* and r = 0.906 *in situ*; $P < 0.05$) between the log permeability coefficients of the model peptides and their respective hydrogen bond numbers (Fig. 2). On the other hand, a correlation coefficient of only 0.389 ($P > 0.05$) was obtained between the log$P_{monolayer}$ of the model peptides and their lipophilicity as measured by the log partition coefficient of octanol–buffer. Similarly, a very low correlation coefficient (0.155; $P > 0.05$) was obtained between the log$P_{in\ situ}$ of the model peptides and their lipophilicity.

While useful for demonstrating the concept, it has been pointed out previously that assigning unit hydrogen bond numbers to the functional groups present in these peptides makes the naive assumption that all the hydrogen bonds are energetically equivalent (8–10). In reality, it is well appreciated that hydrogen bond strength is dependent upon a number of factors such as type of heteroatom involved, along with steric and electronic considerations. Recently, two experimental methods that allow more direct measurement of hydrogen bond or desolvation potential of the peptides have been described (12). One technique, based on previous work by Seiler (11), involves obtaining the difference between the observed log partition coefficients of the peptides determined in a hydrogen bonding solvent (octanol) and a non-hydrogen bonding organic solvent (isooctane). This method assumes that the hydrophobic effects will be similar in both

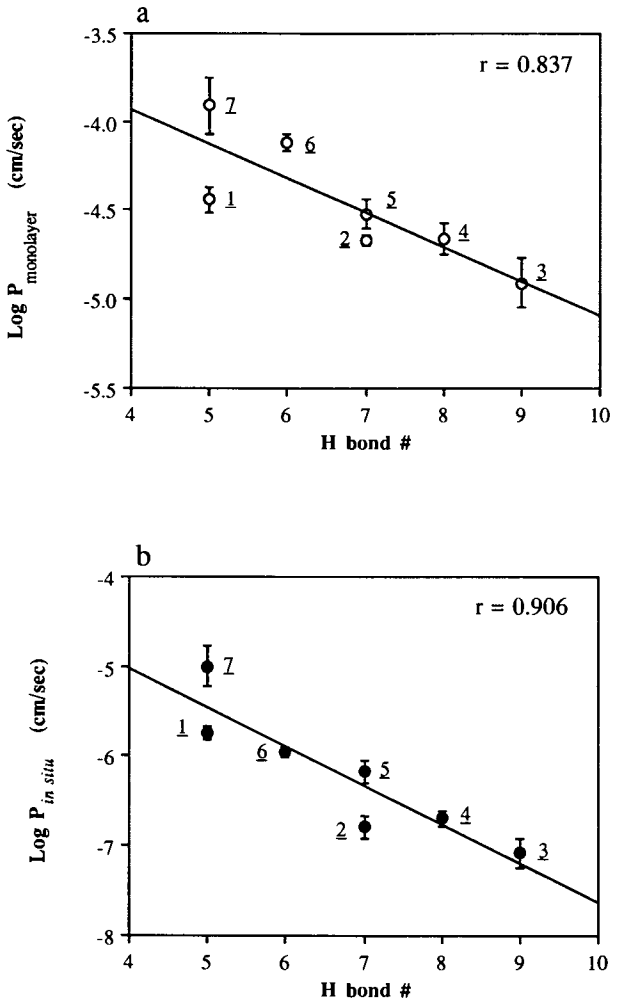

Fig. 2. Correlation between the log permeability coefficients of the model peptides ($P_{monolayer}$ and $P_{in\ situ}$) and their hydrogen bond number (a) *in vitro* and (b) *in situ*. Each point represents the mean and standard deviation for at least three determinations. The hydrogen bond numbers for model peptides were obtained from Refs. 8–10.

organic solvent systems. Therefore, the difference in partitioning must be due to the ability of the organic solvent to accommodate the hydrogen bonding requirement of the solute and is a direct measurement of the hydrogen bond potential (11). The second method used to determine the desolvation energy involves obtaining the partition coefficients of the model peptides in a heptane–ethylene glycol solvent system. It was reasoned that the balance of hydrophobic and hydrogen bonding interactions in this solvent system would be similar to those present in the octanol–isooctane system, and therefore, the partition coefficients should be a direct measure of the hydrogen bond potential (12).

Table I lists the permeability coefficients ($P_{monolayer}$ and $P_{in\ situ}$) for the model peptides together with their respective estimates of desolvation energies $\Delta\log PC$ and $\log PC$ heptane–ethylene glycol. As expected, much better correlations between the log permeability coefficients ($P_{monolayer}$ and $P_{in\ situ}$) and the estimations of hydrogen bond potential than between the log permeability coefficients ($P_{monolayer}$ and $P_{in\ situ}$) and the hydrogen bond numbers were obtained for the model peptides. This was shown by the good correlation coefficients when $\log P_{monolayer}$ and $\log P_{in\ situ}$ were plotted against $\Delta\log PC$ (Fig. 3) ($r = 0.961$ *in vitro* and $r = 0.962$ *in situ*; $P < 0.05$) and the $\log PC$ heptane–ethylene glycol (Fig. 4) ($r = 0.963$ *in vitro* and $r = 0.940$ *in situ*; $P < 0.05$), respectively. Furthermore, these correlations are statistically different from the permeability relationship with the octanol/buffer partition coefficient ($P < 0.05$).

Finally, when the log *in vitro* permeability coefficients and the log *in situ* permeability coefficients for the model peptides were plotted against each other (Fig. 5), a good correlation was observed ($r = 0.928$), suggesting the resemblance of the two different systems toward permeability of the model peptides. On the other hand, the slope of the correlation is 0.445. In theory, if the models were absolutely equivalent, the slope will be one. The deviation from unity suggests that some other factor is present in one model which is not present in the other. One possibility is the not insignificant paracellular flux in the *in vitro* model, discussed previously, which is not found *in situ*. This possibility could be confirmed by either refining the model to minimize this

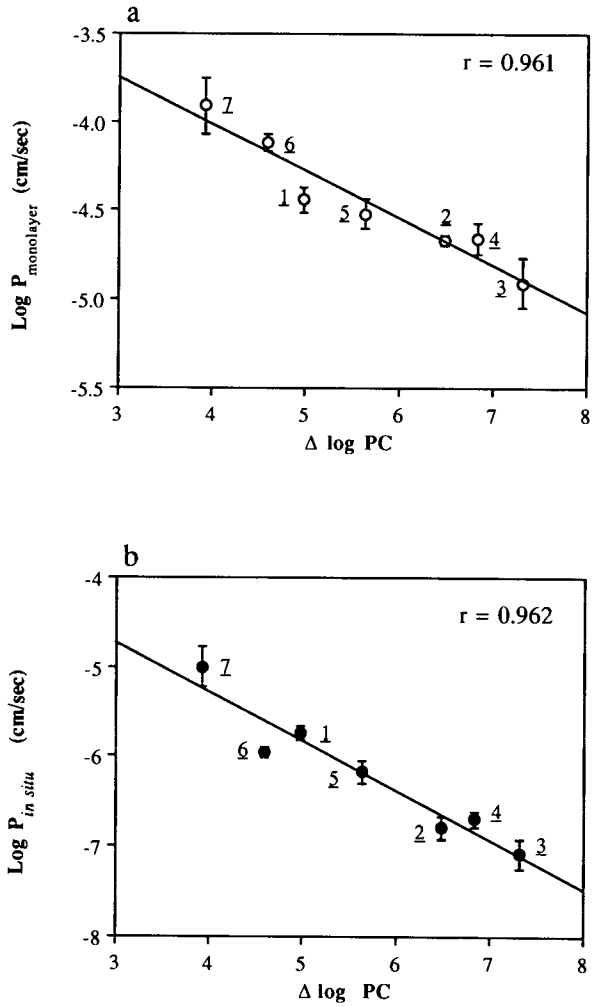

Fig. 3. Correlation between the log permeability coefficients of the model peptides ($P_{monolayer}$ and $P_{in\ situ}$) and their $\Delta\log PC$ (a) *in vitro* and (b) *in situ*. Each point represents the mean and standard deviation for at least three determinations. The $\Delta\log PC$ values for the model peptides were obtained from Ref. 12.

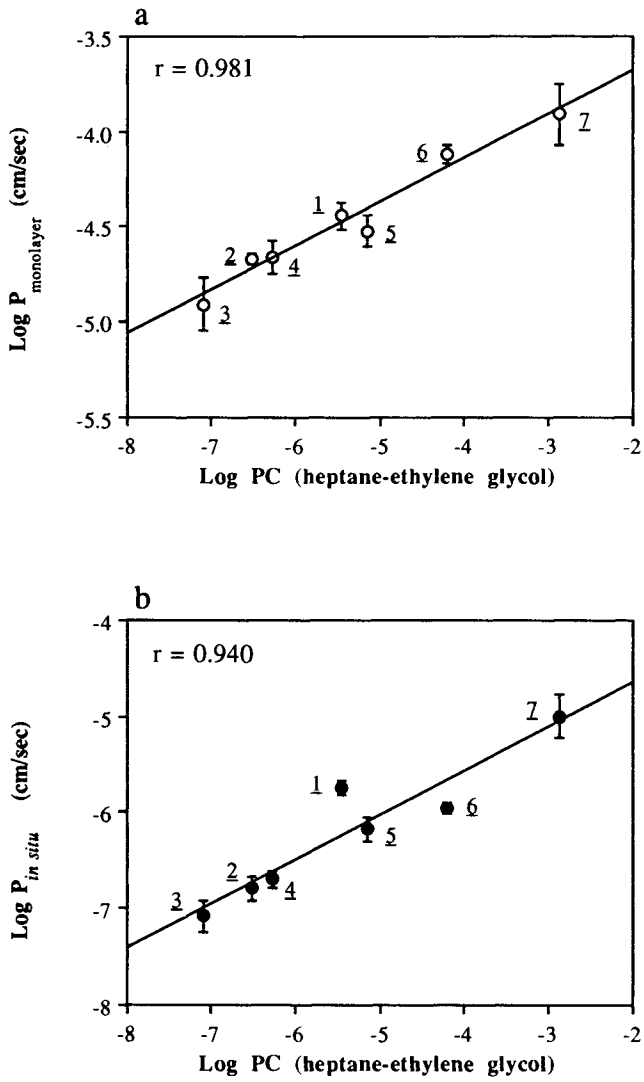

Fig. 4. Correlation between the log permeability coefficients of the model peptides ($P_{monolayer}$ and $P_{in\ situ}$) and their log*PC* (heptane–ethylene glycol (a) *in vitro* and (b) *in situ*. Each point represents the mean and standard deviation for at least three determinations. The log*PC* (heptane–ethylene glycol) values for the model peptides were obtained from Ref. 12.

unwanted flux or correcting the permeability coefficients to obtain $P_{transcellular}$. Both of these approaches are currently being pursued. In spite of this limitation, the good correlation observed confirms the utility of the *in vitro* BBB model to predict qualitatively the *in vivo* BBB permeability of peptides.

In conclusion, the results shown in this study demonstrate that the hydrogen bond potential is a major determinant of BBB transport of peptides and alkylation of the amide bond is an effective method for improving the permeability of a peptide across the BBB. This general conclusion is expected to be applicable to classes of compounds with moderate molecular weight and high desolvation energy. Contrary to previous thoughts, the development of peptide drugs with inherently lower desolvation energies would probably be more useful than an emphasis on high lipophilicity for this compound class.

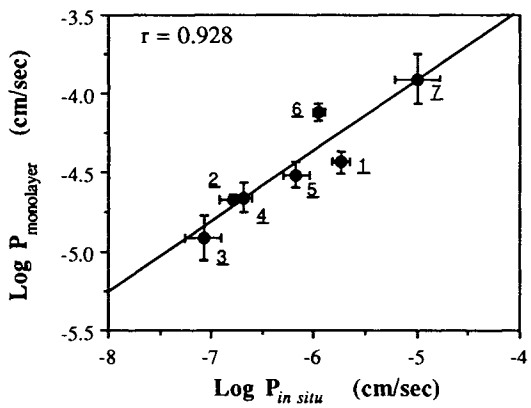

Fig. 5. Correlation between log *in vitro* apparent permeability coefficient ($P_{monolayer}$) and log *in situ* permeability coefficient ($P_{in\ situ}$) for model peptides 1–7. Each point represents the mean and standard deviation for at least three determinations.

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