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Molecular lipophilicity determination of a huperzine series by HPLC: Comparison of C18 and IAM stationary phases

François Darrouzain [a], Philippe Dallet [b], Jean-Pierre Dubost [b], Lhassane Ismaili [a], Fabienne Pehourcq [c], Bernard Bannwarth [c], Myriam Matoga [a], Yves C. Guillaume [a,*]

[a] *Equipe des Sciences Séparatives et Biopharmaceutiques (2SB, EA/3924), Laboratoire de Chimie Analytique et de Chimie Thérapeutique, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon cedex, France*
[b] *Equipe 2962, Laboratoire de Chimie Analytique, 146 rue Léo Saignat, Université Bordeaux 2 Victor Ségalen, 33076 Bordeaux cedex, France*
[c] *Equipe 525, Departments of Pharmacology and Therapeutics, Université Bordeaux 2 Victor Ségalen, 146 rue Léo Saignat, 33076 Bordeaux cedex, France*

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Abstract

Two hydrophobic parameters ($\log k_{w-C18}$ and $\log k_{w-IAM}$, respectively) of a huperzine A series were extrapolated by high performance liquid chromatography (HPLC) using both C18 and immobilised artificial membrane (IAM) columns. A mathematical correlation between C18 and IAM hydrophobic parameters was completed, suggesting a similar behaviour on both columns. This behaviour was principally led by hydrophobic forces. The theoretical lipophilicity ($\log P$) of each compound was computed using Pallas® software and compared to experimental values, showing a similar lipophilic behaviour. Finally, the huperzine $\log k_{w-IAM}$ and $\log k_{w-C18}$ values were correlated with the relative bound percentage of huperzine in human serum albumin, confirming that hydrophobic forces are predominant in the huperzine–HSA binding mechanism.

Keywords: Huperzine; HPLC; Molecular Lipophilicity; IAM; Molecular modelisation

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and cognition. Acetylcholine concentration decreases in the brain of sick persons. One treatment approach is the inactivation of the acetylcholinesterase Enzyme (AchE). This enzyme degrades synaptic acetylcholine. Many medicinal agents, like donepezil or rivastigmine, used for the treatment of AD, belong to the important class of acetylcholinesterase inhibitors [1].

Huperzine A (HupA), a sesquiterpene alkaloid extracted from Huperzia serrata, acts as a potent, highly specific and reversible inhibitor of AchE [2]. Its potency as AchE inhibition is similar or superior to physostigmine, galanthamine, donepezil and tacrine [3].

Even if the importance of drug-membrane interactions in drug design received certainly far less attention than drug–protein interaction, the molecular lipophilicity of small drug molecules has a prominent role in various physico-chemical models describing for example: absorption, distribution, permeability or protein binding [4]. Particularly, drug penetration through various biological membranes is a very important limitative phenomenon on drug efficiency, and this characteristic has a direct impact on its bioavailability [5,6]. One means of evaluating the membrane penetration capacity of various drugs is the determination of their partition properties, which can be used to study different quantitative structure–activity relationships (QSAR) [7]. Chromatography provides an easy, reliable and accurate way to determine the molecular lipophilicity of compounds based on their retention factors. Octadecyl bonded silica stationary phases are often used to determine hydrophobicity [8,9]. However, a simple hydrocarbon chain cannot truly mimic biological membranes. Biomimetic chromatographic partition systems have been recently introduced with immobilized artificial membranes (IAM) as chromatographic packing materials. The first work using IAM columns for HPLC determination of lipophilicity of drug was published by Kaliszan et al. [10] IAM columns, containing a monolayer of phosphatidyl choline

* Corresponding author. Tel.: +33 3 81 66 55 44; fax: +33 3 81 66 56 55.
E-mail address: yves.guillaume@univ-fcomte.fr (Y.C. Guillaume).

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residues covalently bound to silica-propylamine, mimic very closely the fluid phospholipid bilayers of biological membranes [11,12]. Indeed, IAM allowing a combination of hydrophobic, ion pairing and hydrogen bonding interactions, may be a better model to predict drug transport through different types of biomembranes, and then improves our insight into in vivo partition processes. Thus in this study, the hydrophobic behaviour of eight molecules derived from huperzine A has been determined using conventional octadecyl RP-phase chromatography and biomimetic chromatography. The experimental values are compared and correlated with theoretical values to explain their interactions with biological structures (membranes, proteins, ...) and thus predict the bioaviability of HupA.

2. Materials and methods

2.1. Chemicals

Water was obtained from an Elgastat water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. Citric acid and sodium nitrate were obtained from Prolabo (Paris, France). Acetonitrile was provided by Merck (Nogent sur Marne, France). Huperzine A (M1) was obtained from Aldrich (Courtaboeuf, France). Other derivatives were synthesised in the laboratory [11]: *N*-acetyl-huperzine A (M2), *N*-isobutyryl-huperzine A (M3), *N*-benzoyl-huperzine A (M4), *N*-propionyl-huperzine A (M5), *N*-valeryl-huperzine A (M6), *N*-butyryl-huperzine A (M7), *N*-isonicotynyl-huperzine A (M8). Their chemical structures are given in Table 1. General procedure of preparation of huperzine A M8 derivative: huperzine A (100 mg; 0.41 mmol) was dissolved in 5 mL of dichloromethane and 0.41 mmol of isonicotinic anhydride was added. After stirring at 45 °C for 12 h and cooling, the solvent was evaporated under reduced pressure. The residue was dissolved in 15 mL of ethylacetate and washed with 3 × 10 mL of water. The organic layer was dried over sodium sulphate and evaporated. The crude product was purified with preparative thin layer chromatography. Microanalysis data are given in Table 2, 1H NMR is given in Table 3, Melting point and reaction yield were equal to 167 °C and 35%, respectively.

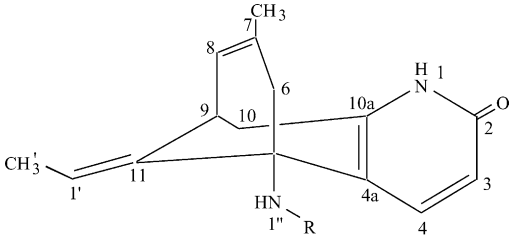
2.2. Apparatus

The chromatographic apparatus was a Hewlett Packard 1090 series 2 equipped with a chemstation A.06.03 software, a DAD detector (Shimadzu, Croissy Beaubourg, France) and an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a 20 μL sample loop.

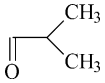
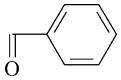
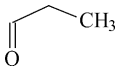
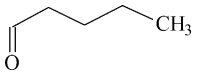
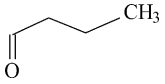
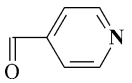
2.3. Chromatographic conditions

Mobile phases were water/acetonitrile mixtures maintained at a flow rate of 1 mL/min. The ODS column (30 cm/3.9 mm i.d.) was prepacked with μBondapak C18, particle size 10 μm (Waters, Milford, MA). The IAM.PC.MG column (15 cm/4.6 mm) was filled with phosphatidylcholine (PC) residues covalently bonded to silica (Regis Technologies Inc., Morton Grove, IL). MG indicates that the silica surface was end-capped with methylglycolate. IAM and C18 columns were used under controlled temperature (25 °C) in an Interchim Crococil oven TM701 (Monluçon, France). Experiments were repeated three times.

Table 1
Structure of the huperzine molecules M1–M8



5-N-*R*-amino-11-ethylidène-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridine-2(1H)-one

R	Name
H	Huperzine A = M1
	*N*-Acetyl-huperzine A = M2
	*N*-Isobutyryl-huperzine A = M3
	*N*-Benzoyl-huperzine A = M4
	*N*-Propionyl-huperzine A = M5
	*N*-Valeryl-huperzine A = M6
	*N*-Butyryl-huperzine A = M7
	*N*-Isonicotinyl-huperzine A = M8

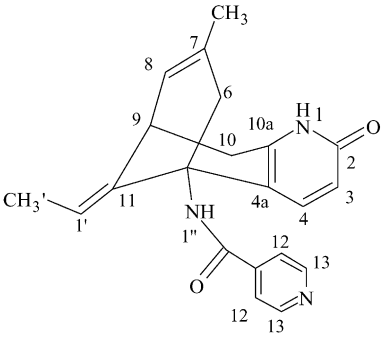
2.4. Methods for lipophilicity determination

The retention time (t_r) of each compound was determined in triplicate on seven different organic modifier-water mobile phase ratios. For each mobile phase composition, the retention factor was calculated according to the formula: $k = (t_r - t_0)/t_0$, where t_0 is the column dead time measured as the retention time of a non retained product (citric acid for IAM and $NaNO_3$ for C18, respectively).

Table 2
Analytical data for M8

	Analytical data calculated	Analytical data found
%C	72.60	72.38
%N	12.10	12.21
%H	6.09	6.17

Table 3
H^1 NMR in $CDCl_3$ for huperzine A M8



^1H	δ (ppm)
H1	4.7
H3	6.65
H4	8.3
H6 and H6′	1.9–2.0
H8	5.66
H9	3.48
H10 and H10′	2.5–2.65
H1′	4.55
CH_3	1.3
CH_3'	1.6
H1″	5.4
H12	9.1
H13	9.2

k_w is the solute retention factor for water as mobile phase and allows to measure experimental molecular lipophilicity. The use of $\log(k_w)$ as a hydrophobic parameter was demonstrated by Hulshoff and Perrin [14]. Usually $\log(k_w)$ values are too high to be obtained experimentally, and therefore have to be calculated using extrapolation techniques.

According to the solubility parameter concept [15], the relationship between solute retention and mobile phase composition can be described by:

$$\log(k) = A\varphi^2 - S\varphi + \log(k_w)$$

where A and S are constants for a given solute–eluent combination and φ is the volume fraction of organic modifier.

Values of $\log(k_w)$ were obtained from the y-intercept of polynomial regression of $\log(k)$ versus organic modifier percentage in the eluent (φ). Related experimental lipophilicity was estimated through $\log k_{w-C18}$ and $\log k_{w-IAM}$ values for C18 and IAM stationary phases, respectively.

Computed lipophilicity was given through calculated/predicted $\log P$ from Pallas software (Pallas 2.0, CompuDrug-Chemistry Ltd.) with ATOMIC database.

3. Results and discussion

In agreement with literature [16,17], $\log k$ values decrease with the increase of acetonitrile percentage in the bulk solvent. This decrease is not linear and has a polynomial shape for both columns. In our study, the polynomial correlation of $\log k$ versus φ displays r values ranging from 0.977 to 0.999. The presence of acetonitrile in the mobile phase is necessary to prevent retention times to be too long. Therefore, the theoretical retention factor for pure aqueous conditions (k_w) is derived from the polynomial extrapolation at $\varphi = 0$ of plots of $\log k$ (measured at different acetonitrile concentrations) versus φ. This extrapolation method was needed for all the studied compounds on the ODS column and for M3–M8 on the IAM column.

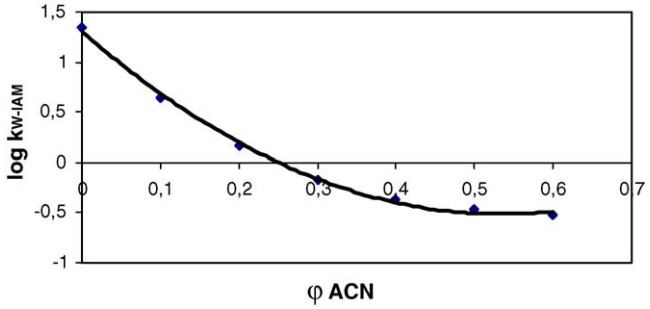


Fig. 1. Plot of $\log k_{w-IAM}$ vs. acetonitrile fraction φ (v/v) for huperzine M1.

In order to verify that extrapolation of polynomial regression allows to make a good estimation of $\log k_w$, experiments for $\varphi = 0$ were carried out on the IAM column for huperzine M1 and M2. The data for huperzine A M1 are presented in Fig. 1. Extrapolated and experimental values of k_w are similar (difference <1%), polynomial regression has therefore been used to extrapolate $\log k_w$ values. The values ($\log k_{w-C18}$ and $\log k_{w-IAM}$, respectively) obtained for the eight huperzine derivatives on IAM and C18 column are presented in Table 4.

The corresponding $\log P$ values derived from an atomic fragment database using Pallas® software are also presented in Table 4. This method allows taking into account interactions like delocalisation and hydrogen binding. However, the system cannot predict electrostatic interactions. The plots of $\log k_{w-C18}$ and $\log k_{w-IAM}$ versus $\log P$ present similar and good regression correlations (over 0.98). They are presented in Figs. 2 and 3. These results were confirmed by the linear relationship between $\log k_{w-C18}$ and $\log k_{w-IAM}$ values (r over 0.99), presented in Fig. 4. This latter result should be confirmed by a similar investigation with a larger series of huperzine derivatives.

This significant correlation could suggest that the retention behaviour is similar for all the huperzine derivatives studied on both column types. They may mainly interact with the lipophilic part of IAM and C18 chains.

Table 4
Values of experimental lipophilicity ($\log k_{W-IAM}$ and $\log k_{W-C18}$) and computed lipophilicity ($\log P$) for huperzine M1–M8

	$\log k_{w-C18}$	$\log k_{w-IAM}$	$\log P$	pK_a "Pallas®" (pK_{a1}/pK_{a2})	b (%)
Huperzine M1	2.2572	1.3037	1.3	11.71/6.99	31.52
Huperzine M2	2.0089	1.1544	0.82	11.51	17.94
Huperzine M3	3.11	1.9841	1.86	11.41	36.19
Huperzine M4	3.977	2.4943	2.84	11.32	53.42
Huperzine M5	2.5421	1.5639	1.66	11.32	31.35
Huperzine M6	3.4777	2.1742	2.38	11.41	42.37
Huperzine M7	3.0129	1.8029	1.86	11.4	39.37
Huperzine M8	2.3389	1.49	1.47	11.26/3.94	x

Relative bound percentage (b) for huperzine M1–M7.

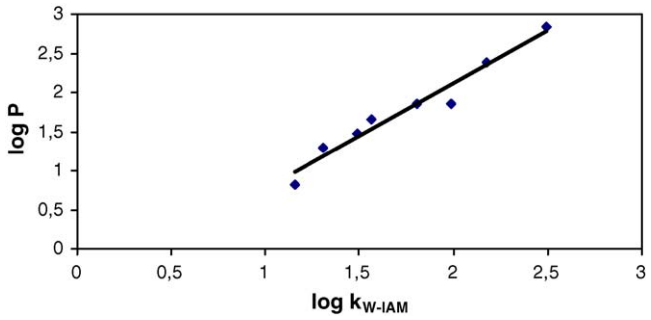


Fig. 2. Plot of log P vs. log k_{w-IAM} for the eight huperzine molecules.

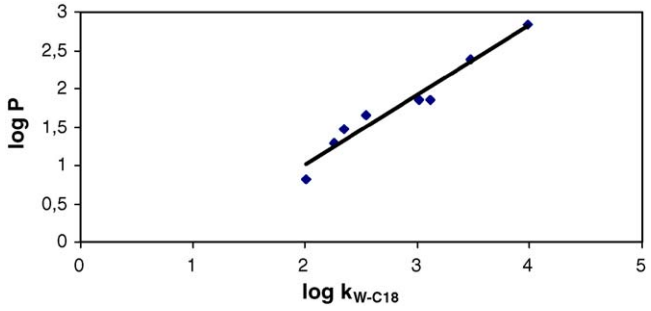


Fig. 3. Plot of log P vs. log k_{w-C18} for the eight huperzine molecules.

As illustrated in Fig. 5, the lipophilicity values log k_{w-C18} for each huperzine derivative are clearly larger than log P and log k_{w-IAM} but they are all arranged in the same order of molecular lipophilicity. The log k_{w-IAM} values are very close to the log P ones. The database of Pallas® software being based on molecular lipophilicities obtained from octanol/water model, computed log P like IAM encodes both polar and hydrophobic forces for lipophilicity determination. This figure also shows that log k_{w-IAM} values are less scattered than log P and log k_{w-C18} suggesting that the IAM stationary phase could be less discriminating than the C18 one. Both columns contain non-polar packing material but the IAM stationary phase with phosphatidyl chain analogues offers polar heads as first contact site for solutes [18]. This difference could explain why log k_{w-IAM} values were found to be lower than log k_{w-C18} values. Consequently the apolar compounds studied have a stronger interaction with C18 than IAM stationary phase [19].

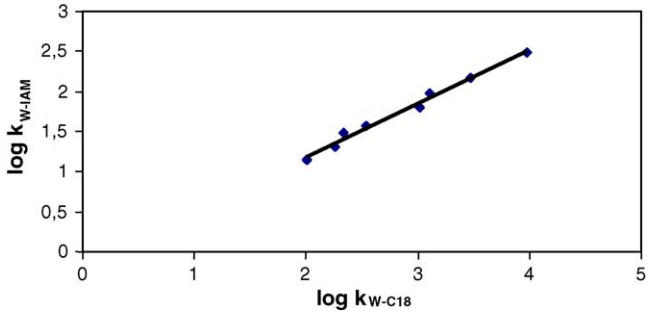


Fig. 4. Plot of log k_{w-IAM} vs. log k_{w-C18} for the eight huperzine molecules.

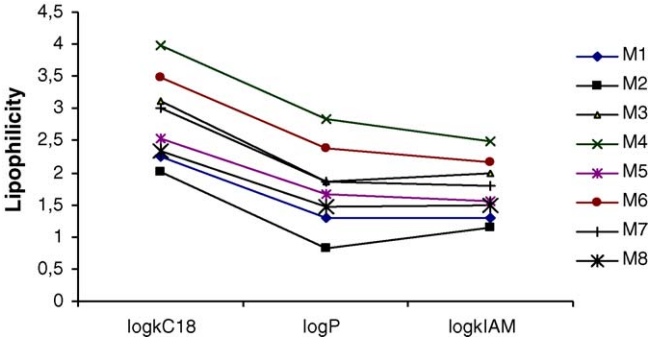


Fig. 5. Comparison between experimental (log k_{w-IAM} and log k_{w-C18}) and computed lipophilicity (log P) values.

Whichever the determination method of lipophilicity, the values increase as follows: M4 > M6 > M7 ≥ M3 > M5 > M8 > M1 > M2. The highest molecular lipophilicity is found for huperzine M4, which is undoubtedly due to non-polar *N*-benzoyl substitution. For M6, M7, M3, M5 and M2, the lipophilicity decrease is correlated to the carbon atom number decrease. The molecular lipophilicity of M1 could be explained by its ionization constant. The different ionization constants of the huperzine molecules studied were calculated using Pallas® software. The pK_a values obtained are given in Table 4. Except for huperzine M1 and M8, all the solutes show a single pK_a value (pK_{a1}), which is associated to acid function. The pK_{a2} of M1 is confirmed by literature [16] and is assigned to amine group basic function. For all the compounds, pK_{a1} values are similar and identical ionization behavior is expected since the hydrogen atom on the pyridone cycle has a very low mobility at the working pH ($5 < pH < 6$). Finally in the analysis conditions, only M1 was in ionized state which explains its low log P and log k_w observed values. Huperzine M2 shows the lowest lipophilicity. Indeed the amide group of all the substituted huperzines is more polar than amine function of huperzine M1. Nevertheless the polar character of amide function is probably shielded on huperzine M3, M5, M6 and M7 by the more lipophilic fragments substituted than the short methyl fragment of M2. Moreover, huperzine M8 shows a lower lipophilicity than M4, suggesting that nitrogen atom of isonicotynyl substitution highly reduces apolar character of M8 compared to benzoyl substitution of M4.

As drug transport across membranes, protein binding (to HSA for instance) depends on different physicochemical properties of the molecules (as molecular lipophilicity, ionisation constant, ...). The relationship between molecular hydrophobicity parameters (log k_{w-IAM} and log k_{w-C18}) and the relative bound percentage (b) to HSA [20] calculated in a previous work [13] for huperzine A M1–M7 was studied for nearly physiological pH of 7. The relative bound percentage (b) has been calculated at 25 °C using the retention factor (k_{HSA}) on HSA for each compound according to the following equation and presented in Table 4:

$$b = \frac{k_{HSA}}{1 + k_{HSA}} \times 100$$

This equation has been shown to give a good correlation versus reference methods for compounds with medium-to-strong binding to HSA [21]. An item that must be kept in mind for the calculation is that they are generally performed with only a small amount of solute. This creates a situation in which a large excess of protein is present and the relative degree of solute bind-

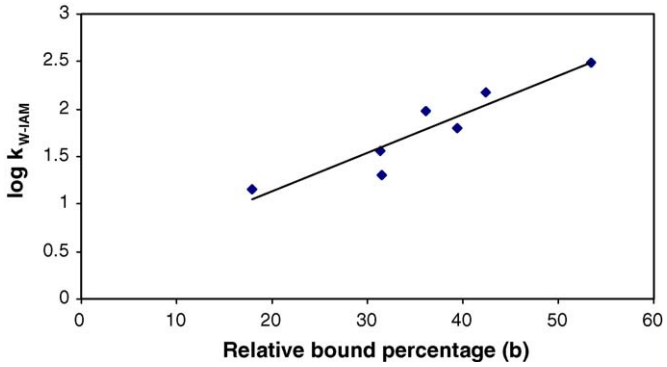


Fig. 6. Plot of $\log k_{w-IAM}$ vs. relative bound percentage (b) for M1–M7.

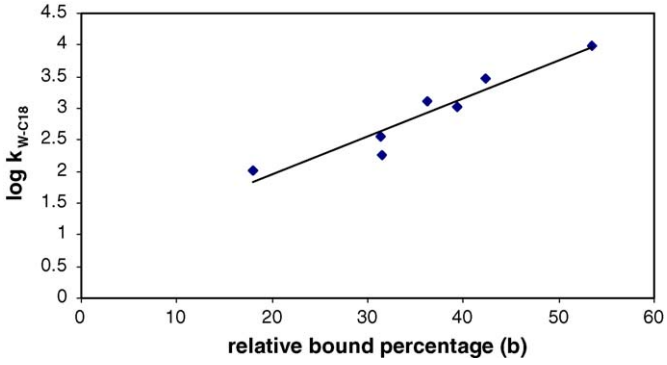


Fig. 7. Plot of $\log k_{w-C18}$ vs. relative bound percentage (b) for M1–M7.

ing is independent of both solute and protein concentrations. The same type of measurement could, in theory, be used for drugs at concentrations that approach or exceed those of their binding proteins; however, in this situation, the results will be dependent on both solute and protein levels, making it essential to know or determine the amount of active protein in the column. Also, care must be taken when dealing with drugs that have multisite binding on a protein, especially when these sites have different susceptibilities to loss of activity during immobilization.

As illustrated in Fig. 6, a significant linear relationship correlating the HSA relative bound percentage to IAM retention parameters taken as a measure of lipophilicity ($\log k_{w-IAM}$) was obtained for compounds 1–7 (correlation coefficient higher than 0.93). As expected, a similar significant [22] correlation (r upper than 0.93) was obtained with $\log k_{w-C18}$, illustrated in Fig. 7. This result suggests that interaction of HupA with HSA may principally be caused by hydrophobic forces. This explanation is corroborated by previous thermodynamic results, which showed predominant hydrophobic forces between HSA and HupA molecules [13].

4. Conclusion

The measured experimental molecular lipophilicities ($\log k_{w-C18}$ and $\log k_{w-IAM}$) are similar and self-consistent. The comparison between the values of $\log k_{w-C18}$ and $\log k_{w-IAM}$ suggests that the same retention behavior between HupA molecules and C18 or IAM stationary phases is involved, with predominant hydrophobic forces. This result is confirmed by good correlation between experimental and computed lipophilicities. Computed molecular lipophilicity cannot take into account electrostatic interactions and thus tends to prove that they are neglected in the experimental molecular lipophilicity determination, suggesting that C18 and IAM hydrophobic parameters can well predict the lipophilicity of huperzines. Finally, the proportionate relationship found between the relative bound percentage to HSA and hydrophobic parameters, seems to confirm that HSA binding is principally controlled by hydrophobic forces.

Acknowledgments

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