56. Isotope Effects on the Lipophilicity of Deuterated Caffeines[1])

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In the present study, it is confirmed that the deuteration of C–H groups is accompanied by a small but genuine decrease in lipophilicity. The lipophilicity of deuterated isotopomers of caffeine was measured by reversed-phase HPLC. Overall, lipophilicity was shown to decrease when going from unlabelled caffeine to the three isomeric trideuterated caffeines, then to the three isomeric hexadeuterated caffeines, and finally to nonadeuterated caffeine. In addition, position-specific effects were also proven. *E.g.* (*7-methyl*-$^2H_3$)caffeine experienced a smaller isotope effect than its two positional isomers. Both a cavity factor (decreased volume of deuterated isotopomers) and intramolecular electronic effects are postulated to operate.

Introduction. – Binding of a ligand to a biological macromolecule, a receptor, or an enzyme is a process comparable in some respects to the extraction of a solute from H_2O into an organic solvent. Thus, the rate and extent of binding of a ligand are related to its lipophilicity [1]. Several physicochemical parameters have been used as an index of lipophilicity, *e.g.* TLC R_m values, reversed-phase HPLC (RP-HPLC) capacity factors ($\log k'$ and $\log k_w$), and partition coefficients measured in H_2O/organic solvent systems ($\log P$) [2].

A convincing body of information [3–10] indicates that deuterium as well as tritium labelling of C–H groups is accompanied by a small isotope effect on the lipophilicity of organic compounds, an effect which despite its smallness may have a marked incidence on binding constants of drugs to biological structures [11]. Using the pairs benzene/($^2H_6$)benzene and toluene/($^2H_8$)toluene, *Cartoni* and *Ferretti* [12] were among the first to show that under RP-HPLC conditions, the deuterated compounds elute faster than their protium isotopomers. A more extensive RP-HPLC study was published simultaneously by *Tanaka* and *Thornton* [13] who, using a range of aliphatic and aromatic hydrocarbons and acids, confirmed the decreased lipophilicity of deuterated analogues, with k'_H/k'_D ratios in the range 1.036–1.076 and a baseline separation of several isotopomers. The RP-HPLC fractionation of labelled drugs and biological molecules from their unlabelled counterparts was also frequently achieved in recent years [14] [15]. In this context, special interest has been devoted to the incidence on chromatographic mobility of pK_a alterations secondary to isotope substitution in the vicinity of ionizable groups [16], occasionally favouring the deuterio analogue in the non-polar stationary phase [17–19]. *Kovach* and

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Quinn [20] reported a 1–2% deuterium isotope effect in the cyclohexane/H_2O partition coefficient of carbonyl compounds, the protium compounds being more lipophilic. This latter study is of significance, since it confirms that the separations observed under RP-HPLC conditions indeed result from a difference in lipophilicity.

Using various aromatic compounds, some of us compared the traditional octanol/H_2O shake-flask method and a RP-HPLC method for their precision in assessing deuterium isotope effects on lipophilicity [21]. While both methods confirmed the decreased lipophilicity of deuterium compounds, the former was far less precise than the latter. We have now extended these studies to investigate a number of deuterated caffeines some of which show RP-HPLC separation [22] [23].

Material and Methods. – The synthesis of the caffeine isotopomers has been described [24]. Unlabelled caffeine (= 3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione), the organic solvents, and all other compounds used in this study were of commercial origin; they were purchased in the highest purity available and used without further purification.

The liquid-chromatography equipment and conditions used for the RP-HPLC determination of lipophilicity were as described [21]. However, a significant alteration in the conditions was the use of a novel lipophilic stationary phase. Indeed, the traditional silica-linked octadecyl phase contains residual free silanol groups which adsorb and retain H-bond-accepting solutes and necessitate the use of a masking agent [25]. Here, we use a *Asahipak-ODP-50* column (150 mm length, 6 mm i.d., particle size 5 μm). This is a polyvinyl alcohol gel esterified with octadecanoyl groups which has proven of particular value for lipophilicity measurements [26]. The flow rate (1.00 ± 0.01 ml/min) was strictly controlled using a digital flowmeter (*Phase Separation Ltd.*, Queensferry, GB), and the studies were conducted as indicated either at room temperature (21–22°) or at 30.0 ± 0.2° (thermostated column and eluent reservoir). Retention times (precision 0.01 min) were measured with a *Spectraphysics 4100* computing integrator. The MeOH deflection provided the value of the column dead time.

Caffeine and deuterated caffeine were analyzed individually as 10^{-4} M aq. solns., 20 μl samples being injected into the chromatographic system. The log *k'* values were either determined using a single eluent (fixed H_2O/MeOH ratio) as indicated, or using 6–7 H_2O/MeOH mixtures and linearly extrapolating to 100% H_2O to yield log k_w values. The H_2O in the eluent was buffered to pH 5.0 with 3-morpholinopropanesulfonic acid/0.2M NaOH; pH effects were neglected, since caffeine ionization state is unaffected by working at either pH 5.0 or physiological pH [27].

Results. – *log* k_w *Values.* The log *k'* values of caffeine isotopomers measured at room temperature varied linearly with the H_2O/MeOH ratio ($r > 0.98$) and could be extrapolated to a hypothetical 100% H_2O eluent (log k_w values). This extrapolation is traditionally done to allow comparison between compounds of largely different lipophilicity and even between values obtained from eluents containing different organic modifiers [28]. The results *(Table 1)* show that deuterium isotope effects exist and that differences

Table 1. *log* k_w *Values of Caffeine Isotopomers Measured at Room Temperature by RP-HPLC Using a Polyvinyl Alcohol Octadecanoyl (ODP) Stationary Phase*

Compound	log k_w[a])	Isotope effect	Isotope effect per ^2H atom
caffeine	1.132		
(1-*methyl*-$^2H_3$)caffeine	1.114	– 0.018	– 0.0060
(3-*methyl*-$^2H_3$)caffeine	1.115	– 0.017	– 0.0057
(7-*methyl*-$^2H_3$)caffeine	1.118	– 0.014	– 0.0047
(1,3-*dimethyl*-$^2H_6$)caffeine	1.105	– 0.027	– 0.0045
(1,7-*dimethyl*-$^2H_6$)caffeine	1.106	– 0.026	– 0.0043
(3,7-*dimethyl*-$^2H_6$)caffeine	1.108	– 0.024	– 0.0040
(1,3,7-*trimethyl*-$^2H_9$)caffeine	1.098	– 0.034	– 0.0038

[a]) The log *k'* values were linearly extrapolated to 100% H_2O from 6 to 7 H_2O (pH 5)/MeOH eluents. The average s.d. on these values is 0.009.

become apparent when an isotope effect per deuterium atom is calculated. For the three trideuterated caffeine isotopomers, the mean isotope effect per deuterium atom is -0.0055 ± 0.0007, while it is -0.0041 ± 0.0003 for the hexa- and nonadeuterated caffeine isomers. These differences being minute may be genuine or artefactual, but the uncertainty introduced by the process of extrapolation precludes a meaningful conclusion. To circumvent this difficulty, log k' values were measured as described below.

log k′ *Values.* Under conditions of strict temperature control ($30.0 \pm 0.2°$), log k' values were measured for a H_2O/MeOH 7:3 *(v/v)* eluent. Very good precision was thus obtained, since four successive injections of the same compound yielded retention times which differed by 0.01 to 0.05 min. Statistical analysis of the results *(Table 2)* confirms that each time three protium atoms are substituted by deuterium atoms, a genuine decrease in lipophilicity occurs. More important, the analysis reveals position-specific effects which were suspected from the results in *Table 1.* Indeed, it is apparent from the results of the trideuterated isotopomers that the deuterium isotope effect in position 7 is smaller than that in position 1 or 3.

Table 2. *log* k′ *Values of Caffeine Isotopomers Measured at 30.0 ± 0.2° by RP-HPLC Using an ODP Phase and a H_2O/MeOH 7:3 Eluent*

Compound	log k'[a]) (± s.d.)	Isotope effect (± s.d.)	Isotope effect per ^2H atom
caffeine	0.5151[b]) (± 0.0005)		
(1-*methyl*-$^2H_3$)caffeine	0.5044[b]) (± 0.0011)	−0.0107 (± 0.0016)	−0.0036
(3-*methyl*-$^2H_3$)caffeine	0.5045[b]) (± 0.0008)	−0.0106 (± 0.0013)	−0.0035
(7-*methyl*-$^2H_3$)caffeine	0.5074[b])[c]) (± 0.0007)	−0.0077 (± 0.0012)	−0.0026
(1,3-*dimethyl*-$^2H_6$)caffeine	0.4965[b]) (± 0.0005)	−0.0186 (± 0.0010)	−0.0031
(1,7-*dimethyl*-$^2H_6$)caffeine	0.4968[b]) (± 0.0004)	−0.0183 (± 0.0009)	−0.0031
(3,7-*dimethyl*-$^2H_6$)caffeine	0.5003[b])[c]) (± 0.0005)	−0.0148 (± 0.0010)	−0.0025
(1,3,7-*trimethyl*-$^2H_9$)caffeine	0.4907[b]) (± 0.0003)	−0.0244 (± 0.0008)	−0.0027

[a]) $n = 4$.

[b]) The log k' value of each compound in the $^2H_0$, $^2H_3$, $^2H_6$, or $^2H_9$ groups is different ($P < 0.05$) from the log k' value of each compound in the other groups.

[c]) The log k' value of this compound is different ($P < 0.05$) from that of the two other compounds in the same group.

The results for the hexa- and nonadeuterated isotopomers are more difficult to interpret. If the isotope effects of the groups C^2H_3–N(1), –N(3), and –N(7) are added and compared with the experimental values of the hexa- and nonadeuterated isotopomers, only (1,7-*dimethyl*-$^2H_6$)caffeine behaves as predicted. In contrast, the other derivatives display an isotope effect which is smaller than calculated by 0.0036 ± 0.0010. If genuine, this difference may point to a subtle 'second-order' perturbation by the group C^2H_3–N(3) and/or to perturbation of self-association phenomena [29] (see *Discussion*).

In this context, it is of interest to note that *Gaspari* and *Bonati* [30] have recently shown that in methylated xanthines the lipophilic contribution of *N*-methyl groups as compared to NH groups is highly position-dependent; while CH_3–N(1) markedly contributes to lipophilicity, CH_3–N(3) and CH_3–N(7) have little or no effect. These authors also found that the additivity rule applied only to the 1,7-dimethylation pattern, a result in keeping with the isotope effect in (1,7-*dimethyl*-$^2H_6$)caffeine. Steric and electronic effects were postulated to account for these differences [30] (see *Discussion*).

Discussion. – Lipophilicity is a complex molecular property, and lipophilicity parameters encode two major contributions which are a volume (or cavity) term and polarity terms [2]. The cavity term is related to solvent-accessible surface area and expresses non-directional solute-solvent hydrophobic interactions, while the polarity factors express various interactions of an electrostatic and more directional nature. It appears currently well established that steric/bulk factors play a major role in the observed deuterium isotope effect on lipophilicity. Because C–H bonds are longer than C–^{2}H bonds by *ca.* 0.005 Å, C-deuterated compounds will have a slightly smaller molar volume than their protium isotopomers, thus favouring the former in the polar phase. This is shown in a number of studies using such compounds as deuterated benzene, toluene, cyclohexane, and aniline, the reported isotope effects ranging from 1.003 to 1.013 [21] [31–35].

It can be noted that polarity/polarizability properties are also affected by deuterium substitution [16] [31] [36–39]. Indeed, not only is the C–^{2}H bond shorter than the C–H bond, it is also less polarizable and hence, presumably, generates weaker *London* dispersion forces [20] [32] [33]. In a molecule such as $CH_3C{\equiv}CH$, a permanent dipole exists which is decreased by 0.012 D in $CH_3C{\equiv}C^2H$, while in $HC{\equiv}C^2H$ a permanent dipole is created which does not exist in $HC{\equiv}CH$ [40]. Deuterium isotope effects in NMR [41], in particular changes in ^{13}C-NMR chemical shifts, may also afford some clues. While the isotope effect is essentially of vibrational origin, vibrational changes reflect the steric and electronic influences of the deuterium atom. Thus, isotopic perturbations of hyperconjugation [42] [43] and deformations of hybridization [44] are now substantiated. Such effects on polarity/polarizability cannot be without influence on electrostatic solute-solvent interactions, but the nature of this influence remains to be understood.

In the case of caffeine, the influence of self-association phenomena cannot be straightforwardly dismissed. Indeed, preliminary NMR studies [23] indicate that the capacity for self-association of caffeine is markedly diminished by deuterium labelling. In H_2O solutions (concentration range 0.005–0.1M), the decrease in capacity for self-association was modest in (3,7-*dimethyl*-$^2H_6$)- and (1,7-*dimethyl*-$^2H_6$)caffeine, marked in (1,3,7-*trimethyl*-$^2H_9$)-, (1,3-*dimethyl*-$^2H_6$)-, and (1-*methyl*-$^2H_3$)caffeine, and most pronounced in (1-*methyl*-$^2H_3$)- and (7-*methyl*-$^2H_3$)caffeine. Whether self-association occurs under HPLC conditions (concentration, solvent) is doubtful. And if, as appears probable, caffeine self-association is hydrophobic in nature, then its decrease should increase apparent lipophilicity. It thus appears that self-association phenomena cannot contribute to the observed deuterium isotope effect on lipophilicity. In contrast, isotope effects in self-association phenomena may play a role together with lipophilicity/polarity variations in explaining why the various deuterated caffeine isotopomers are much better ligands of human serum albumin than caffeine itself [45].

In conclusion, there is now ample evidence to prove that deuterium substitution on C-atoms is accompanied by a decrease in lipophilicity that is genuine and, despite its diminutiveness, may in some cases have marked biological consequences (see *e.g.* [45] [46]). A cavity/hydrophobic factor (decreased volume of deuterated isotopomers) seems to account for part of the isotope effect on lipophilicity, but a role for polarity factors is indicated and calls for further investigations.

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