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NMR water proton relaxation in unheated and heated ultrahigh aqueous dilutions of histamine: Evidence for an air-dependent supramolecular organization of water

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ABSTRACT

We measured 20-MHz R1 and R2 water proton NMR relaxation rates in ultrahigh dilutions (range $5.43 \cdot 10^{-8}$ M–$5.43 \cdot 10^{-48}$ M) of histamine in water (Hist-W) and in saline (Hist-Sal), prepared by iterative centesimal dilutions under vigorous agitation in controlled atmospheric conditions. Water and saline were similarly and simultaneously treated, as controls. The samples were immediately sealed in the NMR tubes after preparation, and then code-labelled. Six independent series of preparations were performed, representing about 7000 blind measurements. R2 exhibited a very broad scatter of values in both native histamine dilutions and solvents. No variation in R1 and R2 was observed in the solvents submitted to the iterative dilution/agitation process. By contrast, histamine dilutions exhibited slightly higher R1 values than solvents at low dilution, followed by a slow progressive return to the values of the solvents at high dilution. Unexpectedly, histamine dilutions remained distinguishable from solvents up to ultrahigh levels of dilution (beyond 10^{-20} in Hist-Sal). A significant increase in R2 with increased R2/R1 was observed in Hist-W. R1 and R2 were linearly correlated in solvents, but uncorrelated in histamine dilutions. After a 10-min heating/cooling cycle of the samples in their sealed NMR tubes (preventing any modification of the chemical composition and gas content), all of the relaxation variations observed as a function of dilution vanished, the R2/R1 ratio and the scatter of the R2 values dropped in all solutions and solvents, and the correlation between R1 and R2 reappeared in the Hist-W samples. All these results pointed to a more organized state of water in the unheated samples, more pronounced in histamine solutions than in solvents, dependent on the level of dilution. It was suggested that stable supramolecular structures, involving nanobubbles of atmospheric gases and highly ordered water around them, were generated during the vigorous mechanical agitation step of the preparation, and destroyed after heating. Histamine molecules might act as nucleation centres, amplifying the phenomenon which was thus detected at high dilution levels.

1. Introduction

A large number of physicians prescribe homeopathic remedies to ill persons (about 2% in USA [1] or UK [2] and up to 15% in India [3]). However, the clinical or biological activity of ultrahighly diluted substances, as used in this practice, remains controversial (among the abundant literature see [4–7] for positive meta-analyses of hundreds of studies, [8,9] for criticisms and [10] for refutations). Meta-analyses of 44 physicochemical studies [11] and of 75 in vitro biological studies [12] could demonstrate an effect of high dilutions; but most experiments did not reach the required standard for quality. Various recent physical studies including thermoluminescence [13,14], nuclear magnetic resonance (NMR) [15,16], calorimetry and conductometry [17–19] managed to show perturbations in the solvent of extremely diluted solutions, even beyond the Avogadro limit. It is worth noticing that a journal has devoted a whole issue entitled "The Memory of Water" where this problem was debated from a scientific viewpoint [20]. According to the pharmacopoeia, homeopathic remedies are prepared in atmospheric conditions following a specific iterative centesimal dilution/agitation procedure (with C1 corresponding to a 10^2-fold dilution, and Cn to a 10^{2n}-fold dilution), so that the concentration of the solute rapidly reaches the theoretical limit of molecular presence, around C12. Hence, physicochemical research is particularly difficult, since contaminants or collateral phenomena from the containers or from the atmosphere can become preponderant. Owing to the long-lasting controversy, there is a need for very rigorous protocols and reproducibility, as proposed in our preliminary studies [21,22] and highlighted in [11], implying similarly treated solvents as controls, repeated series with statistical analysis and drastic care for the preparation and storage of the highly diluted solutions. Interestingly, some of these criteria have been employed, too, in physical studies investigating memory effects of water in more conventional systems [23]. A potent way to study high dilutions consists in following the progressive modifications of the NMR relaxation properties of the solutions induced by the serial deconcentration of the initial solution. In our preliminary studies carried out at 4 MHz, we reported for the first time slight increases in the R2/R1 ratio in dilutions,

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Table 1
Mean relaxation rates of histamine dilutions and controls (all dilutions mingled)

	Native samples				Heated samples			
	N	R1 (s^{-1})	R2 (s^{-1})	R2/R1	*N*	R1 (s^{-1})	R2 (s^{-1})	R2/R1
W	125	0.631± 0.007	0.698± 0.032	1.106± 0.048	123	0.643± 0.008	0.678± 0.010	1.054± 0.015
Hist-W	126	0.631± 0.007	0.708± 0.033	1.121± 0.054	125	0.644± 0.007	0.679± 0.008	1.055± 0.014
t-test		ns	$p=0.017$	$p=0.019$		ns	ns	ns
Sal	125	0.625± 0.008	0.693± 0.032	1.108± 0.049	125	0.632± 0.006	0.668± 0.009	1.057± 0.013
Hist-Sal	126	0.624± 0.006	0.691± 0.030	1.107± 0.049	125	0.632± 0.006	0.667± 0.008	1.056± 0.015
t-test		ns	ns	ns		ns	ns	ns

N is the number of samples. Histamine dilutions and controls were compared by the *t*-test which is the most commonly used method to evaluate differences in means between groups. Differences are significant when $p<0.05$. Non-significant differences are expressed by ns.

up to 10^{-15}–10^{-17} M, of silica–lactose in saline, and of manganese–lactose and histamine in water [21,22]. More recently [16], we applied low-field (0.02–4 MHz) proton longitudinal nuclear magnetic resonance dispersion (NMRD) to ultrahighly diluted silica–lactose in both saline and pure water; a slight increase in R1 (expected) was observed in the first studied dilution (10^{-6}) which vanished unexpectedly slowly upon further dilution. Ultrahigh dilutions remained statistically distinguishable from the pure solvent, similarly treated by dilution in itself, up to the 10^{-24} level of dilution, i.e. near the theoretical limit of molecular presence of the initial solutes. The effect was more pronounced in saline than in water. These unexpected findings prompted further investigation, notably in other conditions, in order to rule out artefacts, such as possible interactions of silica with the glass material used for the preparation, or possible misinterpretation of the NMRD data due, for instance, to an unknown dependence of the frequency dispersion on the dilution level. So, the present study was carried out at a fixed frequency of 20 MHz and with histamine as solute, beyond the 4th centesimal dilution, i.e. beyond the known threshold of NMR sensitivity to detect histamine protons or any paramagnetic contaminants of the solute. It will be shown that the variations in R1 observed as a function of ultrahigh dilution in the NMRD study [16] are reproducible with histamine at a fixed frequency, and that these variations totally vanish after heating of the samples.

2. Experimental

2.1. Preparation of samples

The preparation of the samples was carried out by means of rigorously controlled procedures, as in the previous studies [16,21,22]. The dilutions of histamine in water (Hist-W) or in saline (Hist-Sal) were prepared under atmospheric conditions, following an iterative centesimal dilution procedure, through vigorous agitation achieved by

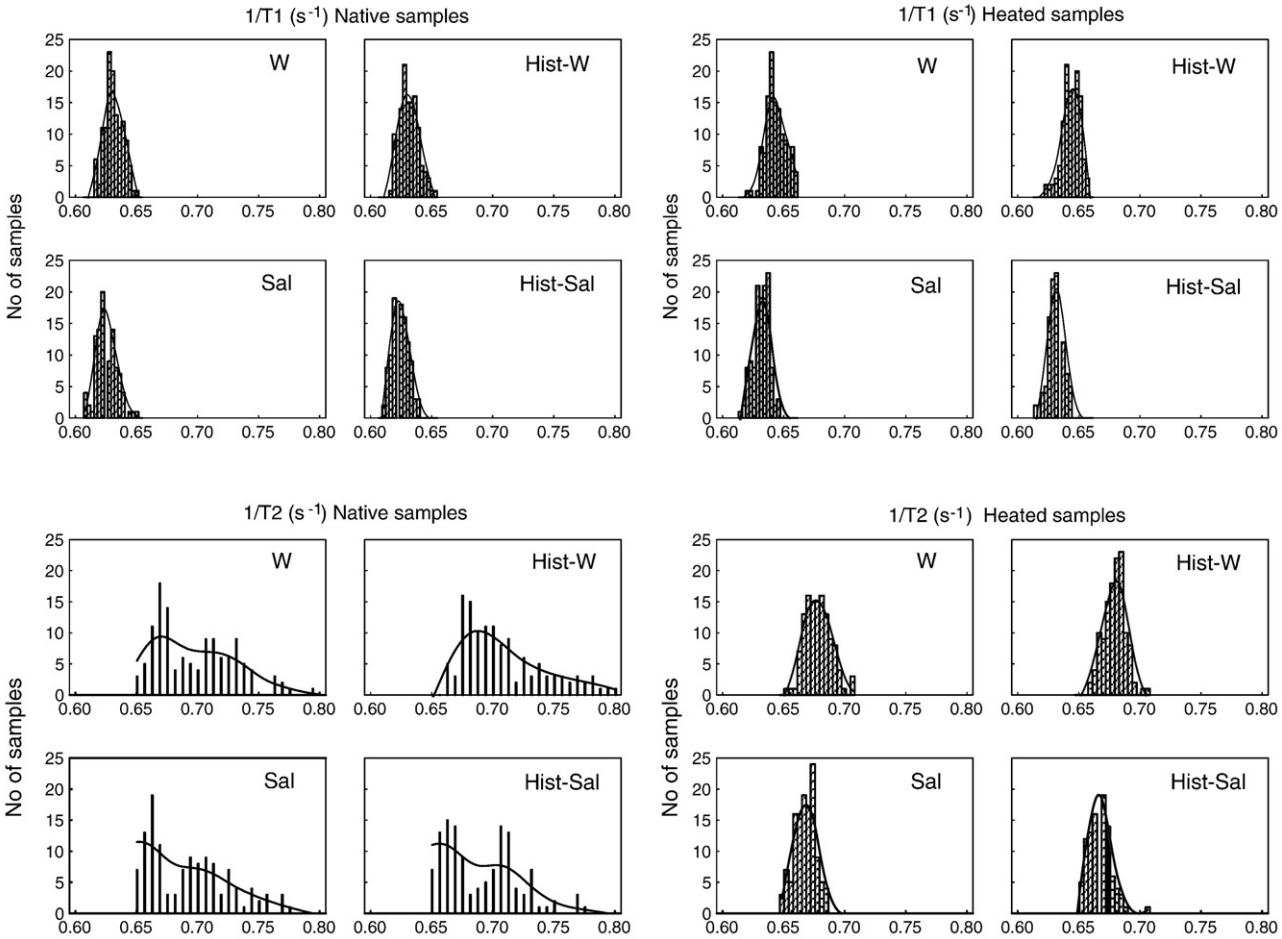

Fig. 1. Histogram distributions of R1 and R2 in native and heated samples (all dilutions mingled). A normal (or Gaussian) distribution of a variable is defined as a monomodal symmetrical distribution centred on the maximum and on the mean value which coincide; it characterizes a homogeneous population. Lilliefors or Shapiro–Wilks tests are commonly used to assess normality of distributions. Here, all distributions were found normal at these tests, except for R2 in the native samples ($p<0.01$) where the distribution looked rather bimodal as emphasized by the applied least-square fitting curve. After heating, the distributions of R2 became normal and significantly thinner (Levene's test devoted to compare the broadness of native and heated distributions; $p<0.001$).

a specific mechanical apparatus manufactured by Boiron for industrial applications which produces 300 violent vertical strokes in 14 s.

2.1.1. Hist-W and Hist-Sal samples

Two hundred milligrams of histamine hydrochloride (Prolabo) was added to 20 ml of water (Biosedra) or to saline (Biosedra) in a 30-ml glass vial under agitation, then submitted to 23 successive centesimal dilutions, by adding 0.2 ml to 19.8 ml of solvent in 30-ml glass vials, using a calibrated dropper. The same dropper was used to fill the NMR tubes and to prepare the next dilution, then was discarded. Dilutions ranging from C4 to C24 were only retained for NMR measurements. The first retained dilution (C4) contained $5.43 \cdot 10^{-8}$ M histamine.

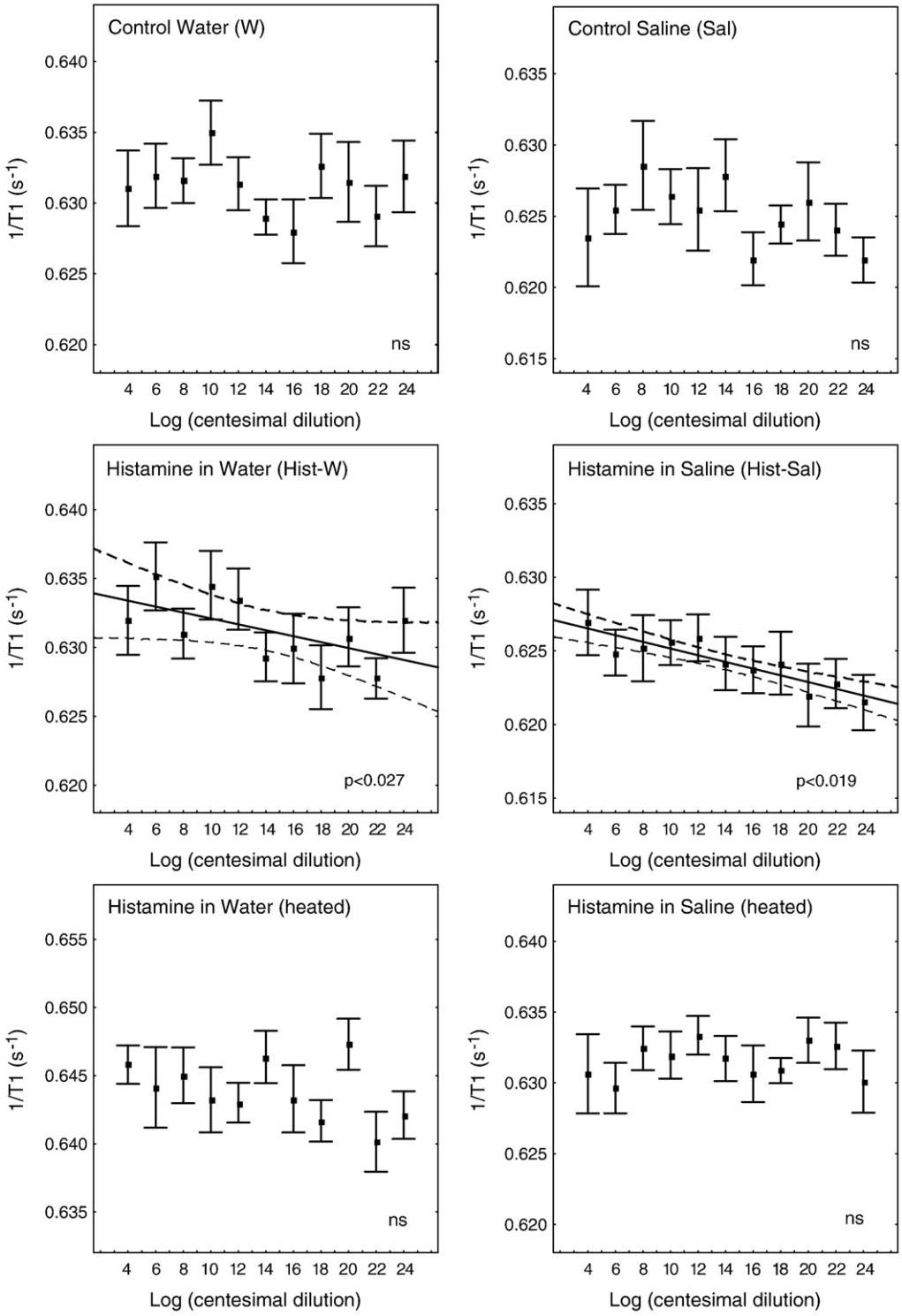

Fig. 2. Relaxation rate R1 in histamine dilutions and in controls as a function of dilution. Data from the six series are plotted with mean value and mean error. Despite the scatter of the values, a trend of variation of R1 is observed in native Hist-W and Hist-Sal, compared to controls and heated samples. This trend required a statistical assessment (see linear correlation analysis — Table 2). Significant linear correlations between R1 and log (centesimal dilution) were confirmed in Hist-W ($p<0.027$) and Hist-Sal ($p<0.019$), expressed by the regression lines (solid lines) reported on the graph with their 95% confidence limits (broken lines). The confidence limits give the range of slopes within which the true slope is located. Controls and heated samples did not exhibit any significant variation as a function of dilution (ns).

Table 2
Linear correlation analysis between relaxation rates and level of dilution

Test of the slope of the relation R1, R2, R2/R1 = *f* [log (centesimal dilution)] (assumed to be linear)									
	Dilution range	C4–C24	C5–C24	C6–C24	C7–C24	C8–C24	C9–C24	C10–C24	C11–C24
R1	Native samples								
	W	ns	ns	ns	ns	ns	ns	ns	ns
	Hist-W	$p<0.027$ (+)	$p<0.024$ (+)	ns	ns	ns	ns	ns	ns
	Sal	ns	ns	ns	ns	ns	ns	ns	ns
	Hist-Sal	$p<0.019$ (+)	$p<0.039$ (+)	$p<0.040$ (+)	$p<0.039$ (+)	$p<0.026$ (+)	$p<0.040$ (+)	$p<0.043$ (+)	ns
R1	All heated samples				No significant relation with dilution				
R2	All native samples				No significant relation with dilution				
	All heated samples				No significant relation with dilution				
R2/R1	All native samples				No significant relation with dilution				
	All heated samples				No significant relation with dilution				

Relaxation rates were arbitrarily expressed as a function of dilution by $R = a + b \cdot \log$ (centesimal dilution), called regression equation, in order to reflect mathematically the trends of variations shown in Fig. 2. Linear correlation analysis is the most simple and the most commonly used test to evaluate the slope *b* of the regression equation. When significant ($p<0.05$), the test means that *b* differs from zero, i.e. that there exists a relationship (correlation) between the relaxation rate and the dilution level. The sign (+) means that the slope is positive. Slopes not significantly different from zero are expressed by ns. The analysis was applied on the whole range of samples (C4–C24), then on more and more diluted samples, up to the Avogadro limit (C11–C24).

2.1.2. W and Sal samples

As controls, water or saline from the same batch was submitted to 24 cycles of centesimal dilution/agitation, by adding 0.2 ml to 19.8 ml under strictly similar conditions as for histamine dilutions. The same C4 to C24 dilutions were retained for NMR measurements.

Six independent series were prepared, at intervals of one month, in order to allow randomization of the atmospheric conditions. All histamine samples and controls belonging to one series were prepared within half a day, in order to avoid barometric fluctuations. For each series, new batches of solvents were used and the order in which the solutions and solvents were prepared was randomly permuted. All operations were carried out under a laminar-flow exhaust hood in sterile conditions, with fresh material, including vials and their plastic caps, glass droppers, NMR tubes, previously cleaned with 70% alcohol of spectroscopic quality and rinsed three times with bidistilled water before sterilization by autoclaving. The temperature variations in the hood were kept within 2 °C during the half-a-day preparation. All these conditions enabled statistical compensation for barometric and temperature fluctuations, likely to influence atmospheric paramagnetic O_2 dissolution, as well as for any contamination from the material, the batches, or the atmosphere. Immediately after preparation of one sample, the NMR glass tube (Bruker NMS PC 7.5 − 180 × 7.5 mm) was precisely filled on a 1.5-cm height of liquid, and flame-sealed in less than 10 s outside the hood. All tubes were code-labelled and randomized before NMR measurements.

2.2. NMR measurements

The proton relaxation rates R1 and R2 were obtained from the reciprocal ($R = 1/T$) of the relaxations times T1 and T2 measured at 20 MHz on a Bruker NMS 120 Minispec. The probe temperature was maintained at 4 °C (better than ± 1 °C) using a continuous circulation of non-protonated cryostatic fluid. Before any measurement, the samples were pre-cooled for 15 min in the cryostatic bath, then placed into the radiofrequency probe and allowed to reach the equilibrium temperature for at least 10 min. The same water sample was used as a reference to control the long time apparatus drift, throughout the entire experiment. The spin-lattice T1 and spin-spin T2 proton relaxation times measurements were carried out using the inversion-recovery sequence (recycle delay: 20 s; real detection mode; 8 points per curve; monoexponential regression fitting) and the Carr–Purcell–Meiboom–Gill sequence (recycle delay: 20 s; magnitude detection mode; 150 points per curve; monoexponential regression fitting).

On an average, NMR measuring generally took place within one and three weeks after preparation of the samples. Each measurement was repeated 7 times, and then averaged. After completion of measurements, the samples were heated for 10 min in a bath of boiling deionized water, rapidly cooled, and measured again in strictly the same conditions. So, a total of about 7000 measurements were performed. A few samples were unavailable due to breakage or loss of liquid despite sealing during the heating step.

2.3. Statistical analysis

The code of the blind protocol was only broken when all the experiments were completed. The statistical tests were performed with the Statistica (Statsoft) software. Conventional linear correlation analysis, *t*-test for independent samples, *t*-paired test, Levene's test (homogeneity of variances), Lilliefors and Shapiro–Wilks tests

Table 3
Linear correlation analysis between R1 and R2

Test of the slope of the relation R1 = *f* (R2) (assumed to be linear)									
Dilution range	C4–C24	C5–C24	C6–C24	C7–C24	C8–C24	C9–C24	C10–C24	C11–C24	C12–C24
Native samples									
W	$p<0.002$	$p<0.004$	$p<0.003$	$p<0.006$	$p<0.010$	$p<0.006$	$p<0.004$	$p<0.005$	$p<0.015$
Hist-W	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sal	$p<0.0001$	$p<0.0002$	$p<0.0002$	$p<0.0004$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0004$	$p<0.004$
Hist-Sal	ns	ns	ns	ns	ns	ns	ns	ns	ns
Heated samples									
W	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$	$p<10^{-6}$
Hist-W	$p<10^{-5}$	$p<10^{-5}$	$p<2\cdot10^{-5}$	$p<0.0002$	$p<0.001$	$p<0.004$	$p<0.011$	$p<0.016$	$p<0.017$
Sal	$p<10^{-6}$	$p<10^{-6}$	$p<2\cdot10^{-6}$	$p<5\cdot10^{-6}$	$p<2\cdot10^{-5}$	$p<10^{-5}$	$p<2\cdot10^{-5}$	$p<6\cdot10^{-5}$	$p<4\cdot10^{-5}$
Hist-Sal	ns	ns	ns	ns	ns	ns	ns	ns	ns

A similar analysis as in Table 2 was applied using the arbitrary regression equation $R1 = a + b \cdot R2$, in order to reflect mathematically the trends of variations shown in Fig. 3. When significant ($p<0.05$), the test means that the slope *b* differs from zero, i.e. that there exists a linear correlation between the two relaxation rates. Non-significant slopes are expressed by ns. The analysis was applied on the whole range of samples (C4–C24), then on more and more diluted samples, up to the Avogadro limit (C12–C24). All slopes were positive (sign (+) not reported on the table).

(normality of distributions) were used and specifically explained in the results section. The significance level (p-value) was set to 0.05. The p-level represents the probability of error in accepting the observed results as valid. In other words, p-levels of 0.05 or 0.001 indicate that there is a probability of 5% or 1‰ that the results are obtained by mere chance.

3. Results

3.1. Mean R1 and R2 in native and heated samples (all dilutions mingled)

The histogram distributions and mean values of R1 and R2 (all dilutions mingled) are reported in Table 1 and Fig. 1. R1 histograms looked normal (Gaussian) in all solutions and solvents, reflecting a rather homogeneous population. On the contrary, R2 exhibited a much broader scatter than R1 in native histamine dilutions as well as in native controls. Moreover, the R2 histograms looked bimodal and differed significantly from Gaussian curves; however, no significant difference in the broadness could be found between controls and histamine solutions, whatever the dilution range. The only significant difference between native histamine solutions and controls was a higher R2 in Hist-W (0.708 s^{-1}) compared to W (0.698 s^{-1}; $p=0.017$), resulting in a higher R2/R1 ratio in Hist-W (1.121 vs 1.106; $p=0.019$). The increased R2 in Hist-W was found uniformly persistent throughout the dilution range (below C7: 0.706 vs 0.696 s^{-1}; above C12: 0.707 vs 0.696 s^{-1}). After heating, significant modifications occurred in both solutions and controls: R1 increased ($p<0.001$ at the t-paired test comparing individually each sample before and after heating), R2 decreased ($p<0.001$) and R2/R1 markedly decreased ($p<0.0001$). Strikingly, the scatter of R2 shortened considerably and became of the order of that of R1; the R2 histograms became normal (Gaussian). The values of R2 in Hist-W became equal to that of W; R2/R1 reached an identical low value (near 1.055) in all solutions and controls.

3.2. Influence of dilution on R1 and R2

The R1 relaxation rates of native and heated samples as a function of dilution are presented in Fig. 2 (the R2 relaxation rates have not been reported since no significant variation was found). Despite a wide scatter of the values which reflected the experimental fluctuations, some trends could be distinguished: no obvious effect of the dilution/agitation process was observed in the controls; on the contrary, Hist-W and Hist-Sal exhibited slightly higher R1 values than controls at low dilution, followed by a progressive return to the values of the controls at high dilution. As discussed in our previous paper [16], these trends could be statistically described by a log-linear regression fitting $R=f$ [log (centesimal dilution)]. The complete results of this analysis are summarized in Table 2; they prove a significant decrease in R1 from low to high dilutions in Hist-W and Hist-Sal samples. The effect was more pronounced in saline, where it was observed up to the C10–C24 range of dilution, i.e. at histamine concentrations lower than $5.43 \cdot 10^{-20}$ M. After heating, all variations observed as a function of dilution in the native samples vanish (Table 2 and Fig. 2).

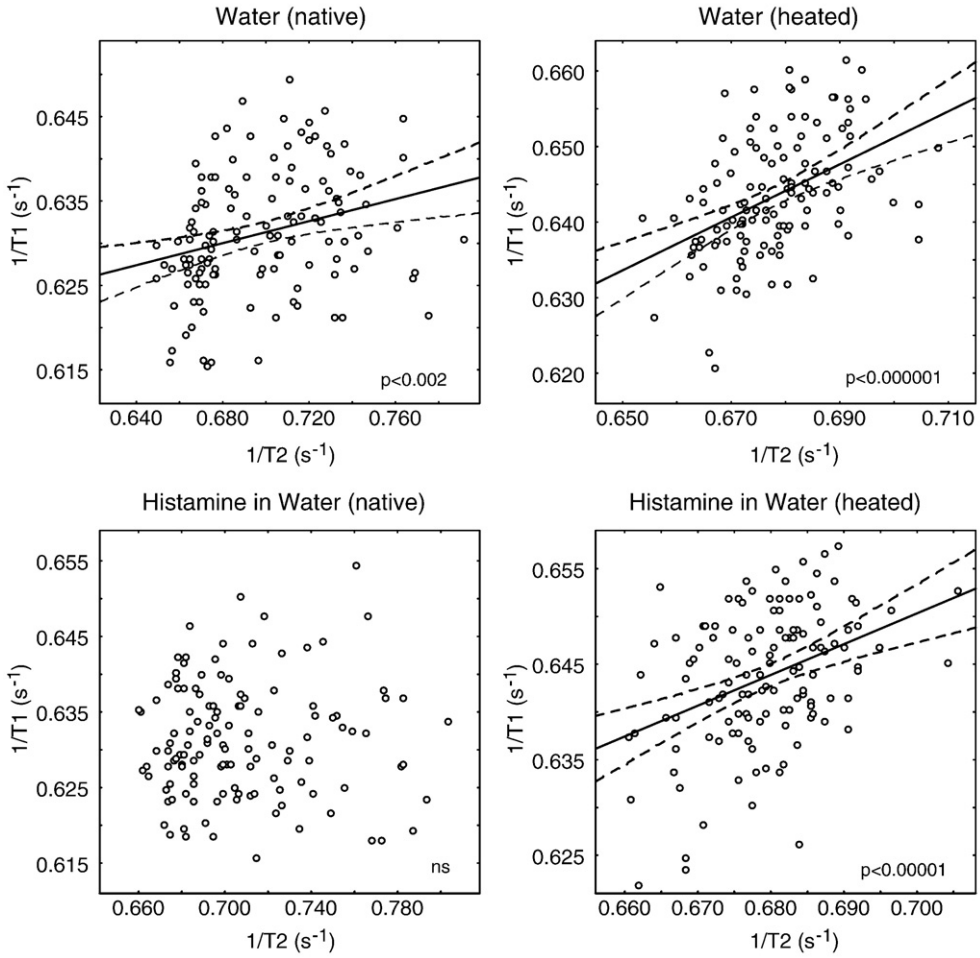

Fig. 3. Correlation between R1 and R2. The figure plots the relaxation rates R1 vs R2 for water (W) and for histamine in water (Hist-W), before and after heating. Very significant linear correlations (see Table 3 for statistical analysis) between R1 and R2 were observed in water and heated Hist-W, expressed by the reported regression lines (solid lines) with their 95% confidence limits (broken lines). No correlation emerged in the native Hist-W samples (ns).

3.3. Correlation between R1 and R2

A linear positive correlation was found between R1 and R2 in the native solvents as well as in the heated solvents (Table 3 and Fig. 3). This means that R1 and R2 varied in a similar and related manner as a function of dilution (see Appendix A). Such a correlation was not observed in the native Hist-W and Hist-Sal dilutions, whatever the dilution range. Strikingly, the correlation reappeared after heating of the Hist-W samples (illustrated in Fig. 3), but not after heating of the Hist-Sal samples. Noteworthy, the correlations were found much more significant after heating.

4. Discussion and conclusion

This study shows modifications of 20-MHz NMR water proton relaxation rates in ultrahigh dilutions of histamine in water and in saline, at dilution levels higher than C4 (10^{-8}), i.e. beyond the sensitivity of the technique to detect the initial solute. Drastic experimental procedures were applied, especially similarly prepared controls and repeated series, and blind measurements, in order to avoid several sources of artefacts. No variation in relaxation rates was observed during the dilution/agitation process applied to the controls. By contrast, histamine dilutions in water and saline exhibited slightly higher R1 values than controls at low dilution, which progressively retrieved the values of the controls at high dilution. This was unexpected at such high dilution levels, but confirmed our previous low-field (0.02–4 MHz) NMRD study [16] carried out on the silica–lactose system; again, it was found to be a more pronounced effect in saline, where the variations in R1 could significantly be observed up to ultrahigh ranges of dilution (C10–C24 here, C12–C24 in [16]). Thus, the phenomenon seemed to depend neither on the nature of the solute, nor on the frequency within the 0.02–20 MHz range, nor on the mode of agitation (linear strokes here, vortex in [16]). Besides, an increased R2 with increased R2/R1 was observed in Hist-W dilutions. According to the theory (see Appendix A), increased R1 and R2 with increased R2/R1 are expected in solutions compared to pure water due to higher organization and/or restricted motion of the water molecules in the hydration layer of the solute [24]. But it seems unrealistic to observe such an effect at very high dilution. However, an increase in R2/R1 in Hist-W was already reported in our preliminary work [22]. At present, these findings remain unexplained and inquire into collateral phenomena (discussed below). In Hist-Sal, no increase in R2, nor no increase in R2/R1 was observed. Saline (0.15 M NaCl) is a much more complex medium than water, making thus even more difficult any attempt of explanation.

Of course, the variations in R1 as a function of dilution in Hist-W and Hist-Sal, shown in Fig. 2, were minute, of the order of the experimental fluctuations. However, they should not be considered as random since they were statistically assessed and have been observed in another laboratory using a different device [16]. Moreover, they were never observed in controls; and several additional findings from the present study argue for real water NMR relaxation modifications induced by the dilution of histamine: i) the variations in R1 as a function of dilution totally vanished after heating, ii) the linear correlation found between R1 and R2 in the solvents vanished in the presence of histamine, then strikingly reappeared after heating of the Hist-W samples, iii) the R1, R2 and R2/R1 values of solvents and histamine dilutions became strictly equal after heating, iv) the R2/R1 ratio of Hist-W dropped after heating, supporting a more organized state in the native samples. It is worth noting that the total equalization of behaviours between pure water and Hist-W dilutions after heating proved a posteriori the absence of any kind of impurities which could have induced these unexpected results.

An attempt of explanation is provided by the heating/cooling cycle directly applied on the sealed NMR tubes, thus preserving their chemical composition and gas content. We postulate that the specific preparation procedure under atmospheric conditions and vigorous agitation induced supramolecular states of dissolved gases and hydrogen-bonded supramolecular water structures, both expected to be destroyed by heating. This assumption corroborates well with the very broad heterogeneity of R2 values observed in all native samples, reflecting a large structural and/or dynamic heterogeneity, which vanished totally after heating. Noteworthy, a structuring effect of atmospheric gases below a critical temperature of 30–50 °C, dependent on the time of exposing to air, and which disappeared after boiling, had already been described by Kondrachuk et al. using NMR T1 proton relaxation [25]. Beside the total cancelling of the heterogeneity of the R2 values, the heating/cooling cycle led to a more significant correlation between R1 and R2 in water, and to a decreased R2/R1 ratio in histamine solutions as well as in solvents, indicating a less ordered structure after heating in pure solvents too, that corroborates Kondrachuk's view of unheated water structured and stabilized by gases. Interestingly, supramolecular structures in high dilutions were suggested from NMR relaxation studies at 600 MHz [15], but unfortunately in a short paper lacking most experimental details on the nature of dilutions and procedure.

Nanosized bubbles have been identified in liquid water [26–29], which are stabilized by traces of ions and tend to associate in fractal clusters, that scatter light. Removal of gases suppresses the small-angle laser-light scattering by water [30]. Radiofrequency(rf)-treatment has been shown to induce formation of arrays of stable (hours) nanobubbles in water and aqueous solutions; degassing of the treated water erases all the effects, and rf-treatment has no effect on degassed water (see [31] for review). The gas–water interface of the nanobubbles is hydrophobic, and therefore the water molecules may form clathrate shells with an "icelike" structure around the nanobubbles [32]. These ordered shells can induce long range structure up to the micrometer level [31]. Let us propose here that nanobubbles are generated during agitation, mostly through cavitation, and induce supramolecular organization of the water molecules in their vicinity, through hydrophobic forces and hydrogen bonding, responsible for the observed heterogeneity of R2. Histamine may act as nucleation centres for nanobubbles clustering, amplifying thus the sub-microscopic heterogeneity of the solution, revealed by means of our statistical analysis up to ultrahigh levels of dilution. By means of a simplified theoretical approach (see Appendix A), one can calculate that larger than 4-nm diameter supramolecular structures with collective orientational motion slower than a few hundred picoseconds, may account for our observations. Such an estimated size is consistent both with the smallest air nanobubbles (d=3.6–4.0 nm) demonstrated in water by small-angle neutron scattering [28], and with the growing evidence for large water clustering, from several tens up to more than 200 molecules in ionic solutions and in water [33–44]. Large-scale long-lived supramolecular structures of water around low molar mass compounds have been shown by laser-light scattering [45,46]. With the same technique, Jin et al. [47] showed that rather stable nanobubbles are implied within the supramolecular structures formed around small organic molecules. According to these authors, bubbles stabilized by small organic molecules could even be a universal phenomenon.

At normal temperature and pressure, dissolved air in water has a concentration of around 1 mM, so the molar gas/histamine ratio grows by a hundred-fold factor at each step of dilution, starting from $2 \cdot 10^4$ in the C4 dilution. This amplifying effect of gases may compensate the deconcentration of the solute and explain the unexpected observation of R1 modifications at ultralow concentrations. The effect was more pronounced in Hist-Sal, where it was significantly observed up to C10–C24. Some other differences were found between Hist-Sal and Hist-W, especially during the heating/cooling cycle, where Hist-Sal looked more stable. This different behaviour of saline could be examined in terms of number, size and stability of nanobubbles taking into account the known stabilizing effect of ions on nanobubbles [26], the enhanced number of small bubbles formed on shaking a salt solution

than pure water [48], and the known inhibition of coalescence of nanobubbles by saline concentrations higher than 0.1 M [48–51].

In conclusion, coming back to the controversy of homeopathy, this study reports physical modifications in the solvent of ultrahigh aqueous dilutions of histamine. It is, of course, an intriguing result, but it is worth claiming, until proof to the contrary, that it might merely reflect a trivial air-dependent phenomenon, or an unsuspected bias, and should not be extrapolated to the so-called "memory of water", often alleged to explain the effectiveness of homeopathy.

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Appendix A

The theory of the NMR relaxation of aqueous solutions is rather complex, but the problem may be considerably simplified here. In concentrations of histamine less than $5.43\cdot10^{-8}$ M the protons of histamine can be neglected so that we are only dealing with the relaxation of the water protons. In pure water the relaxation is essentially governed by dipolar interaction between protons of the same molecule (intramolecular, related to rotational motion) or of two neighbouring molecules (intermolecular, related to translational motion) [24]:

$$\begin{aligned} R1_{intra} &= (1/T1)_{intra} \\ &= 3/20\cdot(h/2\pi)^2\cdot\gamma^4 r^{-6}\{2\tau/(1+\omega_o^2\tau^2)+8\tau/(1+4\omega_o^2\tau^2)\} \end{aligned}$$

$$\begin{aligned} R2_{intra} &= (1/T2)_{intra} \\ &= 3/20\cdot(h/2\pi)^2\cdot\gamma^4 r^{-6}\{3\tau+5\tau/(1+\omega_o^2\tau^2)+2\tau/(1+4\omega_o^2\tau^2)\} \end{aligned}$$

with $\omega_o=2\pi\cdot f$, f the Larmor frequency (20 MHz), γ the proton gyromagnetic ratio, r the intramolecular proton distance, and τ the rotational correlation time. The formula for the intermolecular relaxation rate $(1/T)_{inter}$ is more complicated. Its dependence on the frequency is qualitatively similar but its contribution is around 3-fold lower than the intramolecular relaxation rate [52,53]. So, it will be neglected in order to simplify the reasoning. In pure water at room temperature the correlation time τ is very short, of the order of $2.5\cdot10^{-12}$ s; R1 and R2 are equal. By lowering temperature τ increases, reflecting the reduced motion of molecules; as long as $\omega_o\tau<1$, R1 and R2 both increase but R2 more rapidly, resulting in an increase in the R2/R1 ratio. In that domain, R1 and R2 are "positively" correlated, as they exhibit a similar parallel variation as a function of τ. On the contrary, for long correlation times ($\omega_o\tau>1$) as those present in ice, R1 and R2 begin to diverge (R1 decreases again), leading to a "negative" correlation between R1 and R2. Similar behaviours can be described in solutions. It is well-known that introduction of a solute into water induces organization and reduced mobility of the water molecules in the vicinity of the solute, leading to increased R1 and R2 and increased R2/R1 (positive correlation). In very concentrated systems such as tissues or in macromolecular solutions, bound water is "frozen or icelike", leading to divergent variations of R1 and R2 (negative correlation). Thus, in solutions too, a loss of correlation is expected in the transitional region, where the correlation times of the water molecules lie between 10^{-8} and 10^{-9} s (more precisely about $5\cdot10^{-9}$ s) at 20 MHz. The rotational correlation time of water is given by $\tau=4\pi\eta a^3/3kT$; so, the increase in the correlation time could come from a viscosity change (η term) or, more probably here, from a change in the radius (a term) of the water domain visited by the proton during resonance. For $\tau=5\cdot10^{-9}$ s, the calculation leads to a radius of 2.1 nm. Noteworthy, Tiezzi [15] reached the evidence for supramolecular structures from a similar interpretation of divergent behaviours of T1 and T2 at 600 MHz in high dilutions compared to solvent.

Denoting by f the fraction of water engaged in the hydration of gas and histamine, the experimental relaxation rate can be written as:

$$R_{exp} = (1/T)_{exp} = f\cdot(1/T)_{hydr.} + (1-f)\cdot(1/T)_{bulk}.$$

Owing to the negligible concentration of histamine and to the low molar gas/bulk water ratio, the experimental finding of relaxation changes necessarily implies either a cooperative process of recruiting (increasing f), or a drastic increase in relaxation rates, or both. Highly ordered cluster structures do satisfy these two conditions. Thermal rotation of water molecules in charged $H^+(H_2O)_n$ clusters has been estimated of the order of 10^{-6} s at 25 °C [54,55], similar to those of ice near 0 °C [56]. Besides, collective motion or cooperative orientational motion of water molecules can persist more than 300 ps [57] and over very long distances (≈300 nm [58]), although the orientational memory of individual molecules is quickly lost. From the above equations, with τ lying between 10^{-9} and 10^{-8} s, one can calculate that a $2\cdot10^{-5}$ fraction of highly organized water is sufficient to explain a 30-ms variation (likely to be detected) in the relaxation times.

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