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THE WATER PROTON SPIN-LATTICE RELAXATION TIMES IN VIRUS-INFECTED CELLS

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The water proton spin–lattice relaxation times in HEp-2 cell cultures were determined immediately after 1 h of poliovirus adsorption. The shortening of the water T_1 was closely related to the multiplicity of infection, allowing direct inspections of the virus–cell interaction since the first steps of the infectious cycle. Virus-induced structural and conformational changes of cell constituents were suggested to be detectable by NMR investigation of cell water.

1. Introduction

Although water is a fundamental component of biological systems, its role in cellular structure and function is still a matter of controversy. Nuclear magnetic resonance (NMR) methods have been used extensively to study the properties of water since the relaxation times (T_1, T_2) are dependent on the motional freedom available to the water molecules [1]. Protein solutions, tissues, cell membranes and plants have been frequently investigated [1–4], but the different results from various experimental techniques do not allow a straightforward interpretation.

The relaxation times are shorter in living tissue than in pure water, so a reduced motional freedom of at least a part of the water molecules has been suggested: the controversy is still open about the number of distinct environments and the structure of the water molecules [5–18]. Moreover the relaxation times of tissue water are affected both by the molecular complexity of the tissue and by its water content [19,20] and the question arises about the relationship among the relaxation times, the physiological state of the cell and the water content. Some recent papers [21–24] have outlined the lack of linear correlation between the spin–lattice relaxation time of tissue water and the water content whenever changes in the conformational state of cell constituents occur.

It is known that both spontaneously and virus-transformed cells show alterations of the surface [25–27] which stimulate the immune responses; it is therefore consequent to study such processes by investigating the water spin–lattice relaxation time. Moreover the early steps of virus infection are responsible for membrane changes [28–30]: the virus attachment on the cell receptors results in a modification of the membrane lipid fluidity [31], so looking at the NMR properties of cell water seems a suitable approach also for studying virus infection.

The aim of this paper is to investigate the water proton spin–lattice relaxation times in HEp-2 (laryngeal epidermoid carcinoma) cells infected by poliovirus type 1.

2. Materials and methods

HEp-2 cells (human laryngeal epidermoid carcinoma) [32] were grown in Eagle's minimal essential medium (MEM – Wellcome) supplemented with fetal calf

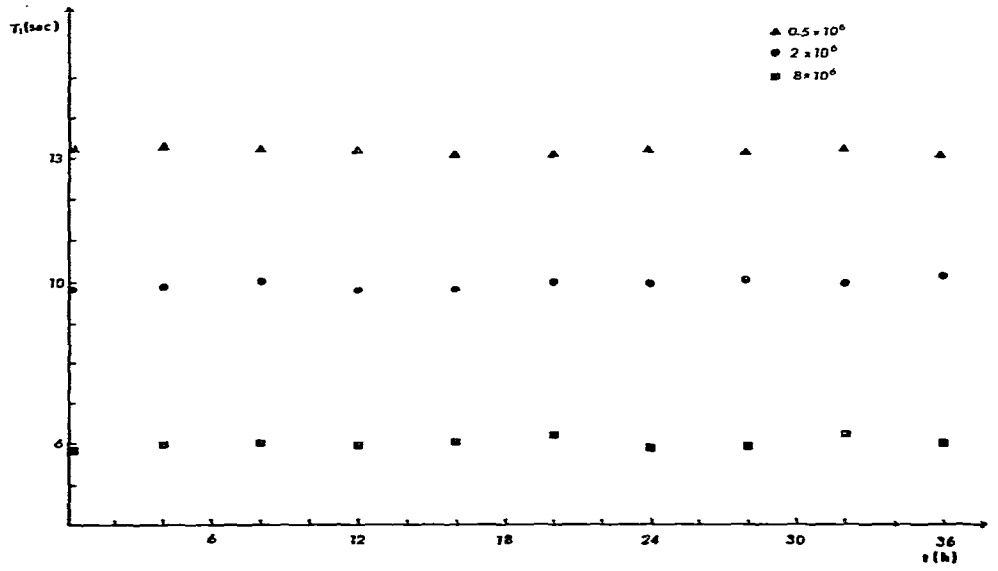

Fig. 1. Spin–lattice relaxation times of cell water protons against the time in hours after the preparation of samples at three different cell concentrations. $T = 37°C$.

serum (Labtek Eurotroph) 10% and penicillin – kanamycin mixture. Poliovirus type 1, LSc 2ab strain [33] was propagated in 37 cells (African green monkey kidney) [34]. Deuterium oxide (D_2O) (99.75% enriched – Merck) was used for the preparation of both the phosphate buffered saline (PBS) solution and the NMR samples; trypsin (Difco) and agar Noble (Difco) were 0.25% in PBS and 3% in bidistilled water respectively.

1 ml of each dilution of a virus preparation was added to a monolayer of 37 cells in a 2 oz flask, after discarding of medium and washing with PBS. The flasks were then placed at 37°C for 1 h and frequently shaken. Eagle's MEM for plaques (without phenol-red) and 3% agar were then added, the medium was left to solidify and the flasks were placed again at 37°C for 48 h. 1 ml of neutral red (Erba) 1/2000 in bidistilled water was added to every flask, the plaques were counted and the titer was given in plaque forming units (PFU).

HEp-2 cells were dispersed by trypsin, centrifugated at 1000 rpm for 20 min at 4°C, resuspended in deuterated PBS, counted, suitably diluted and placed in 5 mm NMR sample tubes. When virus adsorption effects were investigated the cell suspension was homogeneously divided into portions containing about 2×10^6 cells. One of these was taken as a reference standard, the others were mixed with different virus concentrations. All the cell suspensions were incubated for 1 h at 37°C, frequently shaken, centrifugated at 1200 rpm for 15 minutes at 4°C, resuspended in PBS and divided into two identical portions: one of them was placed in the NMR sample tube, the other was suspended in Eagle's MEM and placed in a 2 oz flask. These cell cultures were tested for the appearance of the virus-specific cytopathic effect and for the amount of infectious virus produced.

All the NMR measurements were performed by means of a FT-NMR Bruker WH-90 spectrometer operating at 90 MHz. The resonance frequency of the deuterium nucleus was used as "internal lock". The data were handled with a Nicolet BNC/12 computer having 12 K memory. Spin–lattice relaxation times were measured from the partially relaxed proton spectra obtained from $(180 - \tau - 90 - t)_n$ pulse sequences. The T_1 was evaluated by a least-square analysis of the exponential curve. The error was evaluated at ±5% and the data are the mean value of at least three experiments.

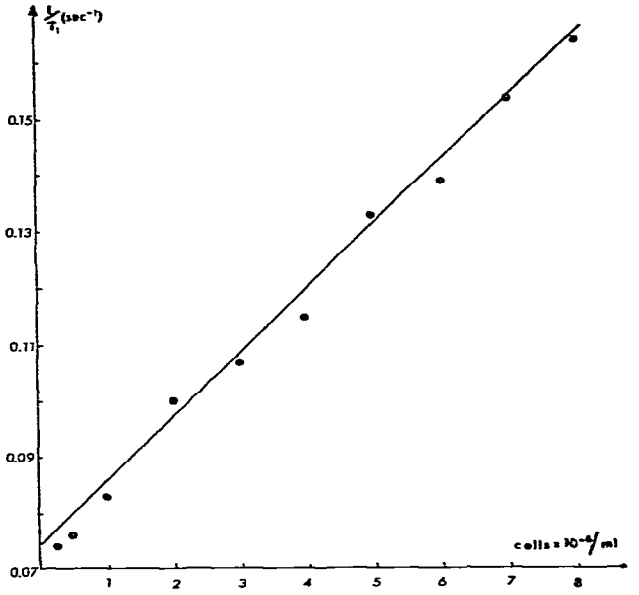

Fig 2. Spin-lattice relaxation rates of cell water protons against the cell concentration. $T = 37°C$.

3. Results and discussion

The water proton relaxation rates are enhanced in tissues and cell suspensions, the extent of the increase depending on the relative weight of internal or vicinal water; namely, pure liquid water at 25°C displays a T_1 of about 2.5 s; whereas the T_1 values for living tissues are of the order 0.25–0.6 s [5,7]. Water dilution with D_2O gives rise to a decrease of $1/T_1$ which is linearly related to the proton mole fraction [35,36]. As a consequence the phosphate buffered saline (PBS) solution in D_2O 99.75% displays a water proton T_1 = 16.4 s, so the detection of proton relaxation rate enhancements due to cellular water is easier in this case than in pure water.

HEp-2 cell suspensions in deuterated PBS display a simple exponential decay of the longitudinal magnetization which fits a single T_1 value. The reproductivity of NMR experiments was tested taking into account the time between the sample preparation and the measurement. Fig. 1 shows the proton spin–lattice relaxation times for three cell samples at different amounts of cells per ml. Each sample was measured immediately after preparation and then every two hours for 36 h (the samples were kept at 4°C during this period). Very little changes are apparent in the limit of the experimental error: the cell samples keep at least 36 h, if placed at 4°C. Moreover, the cell water T_1 is very sensitive to changes in cell concentration.

Fig. 2 shows the concentration dependent longitudinal relaxation rate ($1/T_1$) data: at the lowest end of the curve (about 0.25×10^6 cell/ml) T_1 = 13.6 s; at the highest concentration tested (about 8×10^6 cell/ml) T_1 is reduced over 50%. The linear dependence displayed in fig. 2 with good approximation together with the simple exponential decay of the longitudinal magnetization (unless a time constant $\lesssim 10^{-2}$ s could have escaped detection) points out either a unique environment for the water molecules or, better, fast exchange conditions of water molecules or protons between two or more distinct "phases" of water [8,9, 11,12].

Due to the poor reliability of the cell counting (the error may be evaluated at ±15%) and also because of the possible physiological modifications of the HEp-2 cells, it happened that different T_1 values were found for apparently equal concentrations, so it was not possible to know the T_1 value from the cell concentration or "viceversa". According to these findings it is not possible to compare results from different cell preparations but every experiment must have its own reference control, that is a cell sample strictly homogeneous (see section 2). On the basis of the data reported in fig. 2 a concentration of about 2×10^6 cell/ml has been choosen for the virus infection experiments in order to have relatively high T_1 values with valuable contributions from cell water.

The virus-adsorption experiments were performed by mixing various amounts of poliovirus with a same amount of cell suspension. Table 1 shows the water spin–lattice relaxation times measured immediately after 1 h of virus adsorption. The findings point out a shortening of the water T_1 which is closely related to the multiplicity of infection expressed in terms of the PFU/cell ratio. It is worthy noting that appearance of virus-specific cytopathic effects and production of infectious virus was displayed by all the cell samples but those corresponding to 10^{-6} and 10^{-7} PFU/cell. It is apparent from table 1 that no T_1 shortening is evident at the two lowest multiplicities of infection, so a connection can be suggested between the water proton relaxation rate enhancement and the virus-induced effects.

Table 1
Spin–lattice relaxation times of water in HEp-2 cells (about 2×10^6 cell/ml) one hour after poliovirus infection

PFU/cell	T_1 (s) [a]
b)	13.9
10^{-7}	13.8
10^{-6}	14.0
10^{-5}	13.5
10^{-4}	13.2
10^{-3}	13.0
10^{-2}	12.5
10^{-1}	11.2
1	10.6
10	10.0

a) Mean values of three experiments (±5%)
b) This line refers to the cell control

It must be concluded that cell water T_1 allows direct inspections of the virus-cell interaction since the first steps of the infectious cycle. The feasibility of water NMR relaxation to investigate these cell processes can be ascribed to the detection of very important events at the molecular level, such as the translational and rotational mobilities, the material and non-material exchange processes, the short- and long-range interactions with macromolecular surfaces etc, which probe the structural and conformational changes of cell constituents.

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